Immunization With a Combination of 2 Peptides Derived From the C5a Receptor Significantly Reduces Early Atherosclerotic Lesion in *Ldlr*^{tm1Her} *Apob*^{tm2Sgy}J Mice

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Objective—The goal of this study was to assess whether immunization of *Ldlr*^{tm1Her} *Apob*^{tm2Sgy}J mice with 2 peptides located at the N-terminus of the C5a receptor (C5aR), either alone or in combination, is effective in reducing atherosclerotic lesions.

Methods and Results—Five- to 6-week-old female *Ldlr*^{tm1Her}*Apob*^{tm2Sgy}J mice were immunized using a repetitive immunization multiple sites strategy with keyhole limpet hemocyanin-conjugated peptides derived from the C5aR, either alone (designated as C5aR-P1 [aa 1–21] and C5aR-P2 [aa 19–31]) or in combination (designated as C5aR-P1+C5aR-P2). Mice were fed a high-fat diet for 10 weeks. Lesions were evaluated histologically; local and systemic immune responses were analyzed by immunohistochemistry of aorta samples and cytokine measurements in plasma samples and splenocyte supernatants. Immunization of *Ldlr*^{tm1Her}*Apob*^{tm2Sgy}J mice with these peptides elicited high concentrations of antibodies against each peptide. Immunization with the single peptide inhibited plaque development. Combined inoculation with C5aR-P1+C5aR-P2 had an additive effect on reducing the lesion in the aorta sinus and descending aortas when compared with controls. This effect correlated with cellular infiltration and cytokine/chemokine secretion in the serum or in stimulated spleen cells as well as specific cellular immune responses when compared with controls.

Conclusion—Immunization of mice with C5aR-P1 and C5aR-P2, either alone or in combination, was effective in reducing early atherosclerotic lesion development. The combined peptide is more potential than either epitope alone to reduce atherosclerotic lesion formation through the induction of a specific Treg cell response as well as blockage of monocyte differentiation into macrophages. **(*Arterioscler Thromb Vasc Biol*. 2012;32:2358-2371.)**

Key Words: anaphylatoxin C5a ■ C5a receptor ■ atherosclerosis ■ immunology ■ immune response

The anaphylatoxin C5a, generated by activation of the innate immunity complement component C5, is a potent protein fragment. Binding of C5a to the guanine nucleotide-binding protein (G-protein)-coupled C5a receptor (C5aR) present in immune-inflammatory cells, including monocytes, macrophages, neutrophils, and T cells, leads to proinflammatory activation. C5a exerts its effect by activating the C5aR.[1] The C5aR, also known as complement component 5aR-1 (C5AR1) or cluster of differentiation 88 (CD88), belongs to the rhodopsin-like receptor superfamily, characterized by 7 hydrophobic, transmembrane helical regions connected by 3 extracellular and 3 intracellular loops.[2–6] Among the innate immune components, C5, C5a, and C5aR are abundant and suggested to play critical roles in atherogenesis.[7,8] Atherosclerosis is increasingly recognized as a complex chronic inflammatory disease of the arterial walls,[9–11] as evidenced by the presence of inflammatory cells, activated immune cells, and cytokines in lesions, all of which indicate involvement of the immune system.[12] Monocyte-to-macrophage differentiation and low–density lipoprotein (LDL) oxidation play a pivotal role in early atherogenesis. Macrophages are key players in many aspects of human physiology and disease, including atherosclerosis. By taking up modified LDL (oxidized or acetylated), monocyte-derived macrophages are turned into fat-loaded macrophages residing in the vessel wall, furthering the local inflammatory response and leading to progression of the atherosclerotic plaque. Both innate and adaptive immunities play roles in various stages of atherosclerosis. The binding of C5a to the C5aR is postulated to occur via a 2-site binding mechanism.[13] The basic core of C5a is thought to interact with acidic residues in the receptor amino (N)-terminus,[14] whereas the carboxyl (C)-terminal domain of C5a binds in a pocket formed by largely hydrophobic residues within the transmembrane helices (cylinders 1–7) of the C5aR.[15] In addition, residues between 1 and 35 in the N-terminal domain of C5aR are essential for the interaction with the chemotaxis inhibitory protein of

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Staphylococcus aureus,[16] a 121-residue protein excreted by this bacterium, known to bind to human C5aR[17] and therefore proposed as a new anti-inflammatory agent. In support of this claim, random mutagenesis of the C5aR-N-terminus in the region 19–29 was shown to be relatively highly conservative in the functional mutants.[18] A study with the antiserum of C5aR (aa 9–29) indicated that this antiserum effectively blocks C5a-mediated cell activation, which is because of interference with C5a binding to its receptor by interacting with the extracellular N-terminal region of the receptor.[19] These data provide evidence to show that the N-terminal region of C5aR makes a productive physiological interaction with its ligand. Inhibition of complement C5aR using a peptidomimetic C5aR antagonist or a blocking anti-C5aR monoclonal antibody has recently been reported to limit neointimal formation after wire injury in atherosclerosis-prone ApoE$^{-/-}$ mice.[20] However, the effect of antibodies induced by putative peptide epitopes of C5aR for binding of C5a on atherosclerotic lesion development has not been studied. Therefore, in the present study, we investigated the effects of immunization with 2 peptides derived from the N-terminus of C5aR on atherosclerotic lesion in the *Ldlr*^{tm1Her} *Apob*^{tm2Sgy}J mouse model.

Materials and Methods

Free and keyhole limpet hemocyanin (KLH)–conjugated C5aR-P (aa 1–21 of C5aR), C5aR-P2 (aa 19–31 of C5aR), ApoB peptide, and human HSP60 peptide were synthesized by Severn Biotech Limited (Worcestershire, UK).

Peripheral Blood Mononuclear Cell Isolation From C57BL/6 Background Mice

Blood was diluted with PBS (1:4 dilution) and slowly layered onto preprepared Histopaque solution (1:1 v/v) (Sigma, Dorset, UK) in a centrifuge tube, followed by centrifugation (2000 rpm) at room temperature for 20 minutes to collect mononuclear cells. The isolated peripheral blood mononuclear cells (PBMCs) were used for further flow cytometric analysis for their differentiation.

Animal Experiments

The experiments were approved by the Animal Welfare Committee of the University of Szeged and conformed with the Directive 2010/63/EU of the European Parliament.

Ldlr^{tm1Her}*Apob*^{tm2Sgy}J mice on a C57BL/6 background were used in our study, with a total of 4 groups, each comprising 8–10 females (5–6 weeks old; similar body weight; Table I in the online-only Data Supplement). The immunizing antigens KLH–C5aR-P1 and KLH–C5aR-P2 were injected either alone at a dose of 20 μg per inoculation or the 2 antigens (10 μg each) were combined. Each antigen was mixed with alum adjuvant. Control groups received KLH with alum. The repetitive immunization multiple sites strategy was adopted, and mice were killed at the end of week 12 after being fed a high-fat diet for 10 weeks (the diet was started at week 2 and was continued for 10 weeks). For cross-reactive study, 3 groups of mice (6 per group) were immunized with C5aR-P1, C5aR-P2, and control KLH. Mice were fed normal chow for 5 weeks.

Antibody Response Measurements, Tissue Preparation, Size, and Composition of Atherosclerotic Lesions

Twelve weeks after the first immunization, heart segments with the aortic root were harvested and mounted in an optimal cutting temperature component for immunohistochemical analyses and in paraffin for lesion measurement. Atherosclerosis in aortic roots was examined by Image-Pro Plus software, version 4.0 (Media Cybernetics, Bethesda, MD). The presence of collagen was determined by Sirius Red staining. Longitudinally opened descending aortas were evaluated for the extent of atherosclerosis after Oil Red O staining. Peptide-specific antibody titers were measured by ELISA per the manufacturer's instructions.

Immunohistochemical and Morphometric Analyses and Quantitative Measurements of Atherosclerosis

Sections from optimal cutting temperature–embedded samples were stained with CD68-, CD11c-, CD4-, and forkhead box protein-3 (Foxp3)-specific antibodies. Sections from paraffin-embedded tissues were stained with hematoxylin and eosin and elastin stain (Sigma) for histological examination and evaluated using an Olympus U-ULH Optical microscope (Olympus Optical Co. Ltd, Tokyo, Japan). The extent of atherosclerosis in the aortic roots and descending aortas was measured and analyzed quantitatively by using Image-Pro Plus software version 4.0 (Media Cybernetics).

Measurement of Cytokines

Plasma concentrations of interleukin (IL)-10, transforming growth factor (TGF)-β, tumor necrosis factor (TNF)-α, and interferon (IFN)-γ were measured by ELISA per the manufacturer's instructions (R&D Systems, Abingdon, UK). IL-10 and TNF-α concentrations in the lesions were quantified by immunohistochemical analyses (rat anti-mouse TNF-α and IL-10 were purchased from BioLegend, CA). Concentrations of ConA-induced IL-10, TGF-β, TNF-α, and IFN-γ in splenocyte cultures were also measured.

Antigen-Specific Regulatory Function Assays

For antigen-specific regulatory function, experiments were performed as previously described,[21,22] with some modification. In brief, CD4$^+$CD25$^+$ Treg cells were isolated from spleen CD4$^+$ T cells of *Ldlr*^{tm1Her}*Apob*^{tm2Sgy}J mice immunized subcutaneously with C5aR-P1-, C5aR-P2-, and C5aR-P1+P2.

T-effector cells, the CD4$^+$CD25$^-$ cells, were isolated from CD4$^+$ T cells (unbound to CD4$^+$CD25$^+$ cell beads, 99.5% of CD4$^+$CD25$^-$ cells) from spleen of mice that had received oral immunization with C5aR-P1, C5aR-P2, and C5aR-P1+P2, respectively. CD4$^+$CD25$^-$ cells (2×10^5) were cocultured with CD4$^+$CD25$^+$ cells (2×10^5), in the presence of 2×10^4 antigen-presenting cell (3000 rad irradiated splenocytes) per well, and stimulated with 1 μmol/L C5aR peptides or with KLH control or without C5aR peptides. After 3 days of culture, cells were pulsed with 0.5 μCi of [^{3}H]thymidine (Amersham, Buckinghamshire, UK) for the last 18 hours of culture and then harvested, and the incorporation of [^{3}H]thymidine was determined using a liquid scintillation spectroscopy method (1205 Betaplate, Turku, Finland).

Real-Time Reverse Transcription–Polymerase Chain Reaction Analysis

Total RNA was extracted from the aorta arch using TRIzol reagent (Invitrogen, Carlsbad, CA). Reverse transcription–polymerase chain reaction was performed using a 2-step SYBR superscript reverse transcription–polymerase chain reaction kit (Invitrogen) and using an ABI PRISM 7500 sequence detection system (Applied Biosystems, CA) to analyze the expression of IL-10, TGF-β, TNF-α, and IFN-γ.

Fluorescence-Activated Cell Sorting Analysis

Cells (either PBMC or spleen cells) were processed for staining (30 minutes at 4°C) using phycoerythrin-conjugated CD206 anti-mouse antibody (BioLegend, Cambridge, UK) and fluorescein isothiocyanate-F4/8 anti-mouse antibody (eBioscience, Hatfield, UK). Spleen cells from mice immunized with either C5aR peptides or KLH

(control) were used for Treg measurement using a Treg detection kit (Miltenyi Biotec, Surrey, UK). Cells were analyzed with a Beckman-Coulter FC-500 Analyzer (Beckman Coulter, High Wycombe, UK).

Statistical Analyses

Data are reported as mean±standard error of the mean (±SEM), unless otherwise indicated. Figures were plotted using GraphPad Prism 5.01 and Sigma plot 9.0. For atherosclerotic lesion size, data were compared and intergroup differences were conducted using 1-way ANOVA for multiple comparisons and post hoc Bonferroni test. Other data were analyzed using Student *t* test (2-tailed analyses). Nonparametric distributions were analyzed using Mann-Whitney *U* test for pairwise comparisons and the Kruskal-Wallis test for multiple comparisons. Differences between groups were considered significant at $P<0.05$.

Results

C5a and C5aR Are Involved in Lesion Sites

The results obtained from the tissues of mice immunized with C5aR peptides either alone or in combination showed that C5a or C5aR is expressed in the lesion sites of aorta sinus (Figure IA in the online-only Data Supplement) in mice immunized with different peptides or with KLH only as controls. Although both $C5aR^+$ cells per mm^2 lesion and the C5a percentage of area in the lesion showed somewhat similar profiles between the sampling groups and the control (Figure IB and IC in the online-only Data Supplement), most C5a were not colocalized with C5aR or the number of $C5aR+C5a^+$ cells were decreased significantly in mice immunized with C5aR peptides compared with those in KLH-immunized control mice showing almost all $C5aR^+$ cells combined with C5a (Figure IA magnified, ID, and IE in the online-only Data Supplement). Interestingly, additional tissues of mice immunized with ApoB peptide, human HSP60 peptide either alone or in combination showed that not only C5a or C5aR expressed in the lesions of aorta sinus but C5a colocalized with C5aR (Figure IF–IH in the online-only Data Supplement).

Peptide-Specific Antibody Measurement in the Sera of Immunized Mice, C5a Level in Plasma, and C5aR Levels in Lymph Nodes

After immunization of *Ldlr*^{tm1Her}*Apob*^{tm2Sgy}J mice with KLH-conjugated peptide antigens C5aR-P1 or C5aR-P2, or combination of C5aR-P1 and C5aR-P2, a peptide-induced specific immunoglobulin (Ig)G1 response was observed when compared with either KLH+alum or an alum-only control (Figure 1A). In addition, IgG2c antibody response was detected in the pooled serum of peptide-immunized mice at a low number of dilution times when compared with that in control (Figure 1B). However, the levels of IgG2c detected were much lower than those of IgG1 based on the optical densities measured in different dilutions of samples. Either IgG1 or IgG2c antibody response was significantly high compared with that of the controls. Furthermore, higher levels of anti-oxLDL IgM and IgG were also detected in serum of mice immunized with either C5aR-P1 or C5aR-P2 and fed with normal chow when compared with those of native LDL (Figure IIA in the online-only Data Supplement). In contrast, some degree of anti-C5aR peptide antibodies was also detected in the pooled serum of mice immunized with ApoB peptide (Figure IIB in the online-only Data

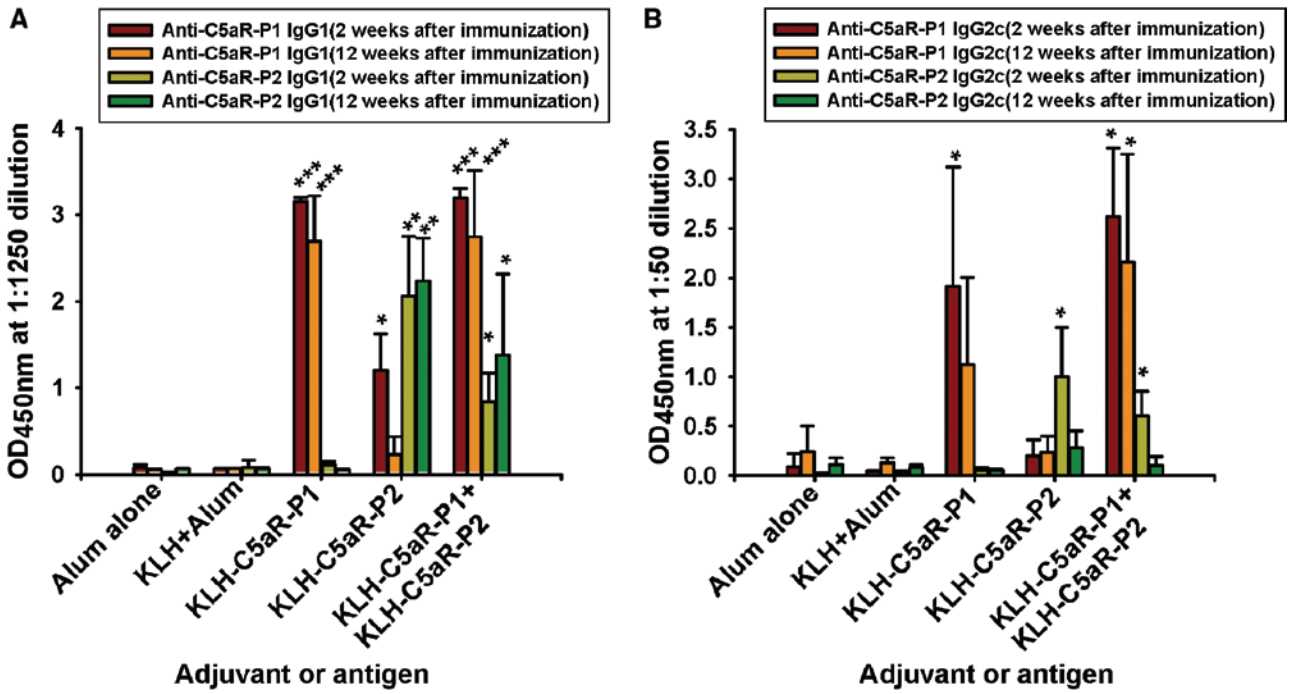

Figure 1. **A**, Levels of peptide-specific IgG1 antibodies in the sera of *Ldlr*^{tm1Her} *Apob*^{tm2Sgy}J mice at 2 and 12 weeks after the first immunization and in controls (alum alone and keyhole limpet hemocyanin [KLH]+Alum-immunized mice). The mean optical densities (ODs) ±SEMs from 6 mice (*$P<0.05$, **$P<0.01$, and ***$P<0.001$), compared with above 2 corresponding controls, that were considered statistically significant are shown. Dilution ratio: 1:1250. **B**, Levels of peptide-specific IgG2c antibodies in the sera of *Ldlr*^{tm1Her} *Apob*^{tm2Sgy}J mice mice at 2 and 12 weeks after the first immunization and in controls. The mean ODs from 6 mice (*$P<0.05$), compared with 2 corresponding controls, that were considered statistically significant are shown. Dilution ratio: 1:50.

Supplement). The immunization with C5aR peptide did not alter C5a level (Figure IIC) and oxLDL level (Figure IID in the online-only Data Supplement) in the plasma and C5aR expression level in lymph nodes from C5aR peptide-immunized mice, which showed similar levels to those from KLH-immunized mice (Figure IIE in the online-only Data Supplement).

Reduction of Atherosclerotic Lesion Size in Aortic Sinus and in Descending Aorta

Representative sections of aortic sinuses from experimental groups are shown in Figure 2A. Lesion size was significantly smaller in mice immunized with either C5aR-P1 or C5aR-P2 (both $P<0.001$) compared with the KLH-alum–immunized control group, either by regarding the mean lesion area (384 710±24 186 μm^2) or the percentage of the total area occupied by lesion (45%±1.9%). Although there was a significant difference in lesion size between mice immunized with C5aR-P1 (221 415±5377 μm^2) and C5aR-P2 (198 931±9196 μm^2; $P=0.035$), there was no significant difference between these 2 peptide antigens in terms of the percentage of the lesion versus total area in the aorta sinus (27.3%±1.4% for C5aR-P1 and 30.2%±0.81% for C5aR-P2; $P=0.08$) (Figure 2B and 2C). The smallest lesion area was observed in mice immunized with the combination of the 2 peptides (171 480±9276 μm^2 based on lesion area; $P<0.07$ to $P<0.001$) when compared with the single peptide–immunized mice. With respect to the percentage of the lesion area versus total area in the aorta sinus (Figure 2C), the difference was significant only between the combined peptide and the C5aR-P2, showing 25.3%±1.2% versus 30.2%±0.81% ($P<0.001$). Reduction in lesion size, expressed as a percentage of the decrease in the lesion size in the aortic sinus of peptide-immunized mice calculated with respect to the mean lesion size in the KLH-alum-immunized control mice, was significant ($P<0.001$), showing a 39.3% reduction (27.3% versus 45%) after immunization with C5aR-P1 and a 32.7% reduction (30.28% versus 45%) with C5aR-P2 (Figure 2C). Mice immunized with the combined peptides showed a significant reduction (25.3% versus 45%) in lesion size (43.7%; $P<0.001$), which was also significant when compared with the mice immunized with C5aR-P2 ($P<0.001$) (Figure 2C).

We also examined the impact of treatment with C5aR peptides on the collagen in these lesions. The reduction of atherosclerosis in mice treated with these peptides was associated with an increase in collagen content: 2-fold (46 061±10 138 μm^2 versus 23 536±3890 μm^2; $P=0.018$) for C5aR-P1, 1.8-fold (42 859±4936 μm^2 versus 23 536±3890 μm^2; $P=0.016$) for C5aR-P2, and 3-fold (71 075±13 816 μm^2 versus 23 536±3890 μm^2; $P=0.001$) for the combined peptides (Figure 2D and 2E). Mice immunized with the combined peptides showed a significant collagen increase compared with mice immunized with the peptides alone ($P=0.033$, and $P=0.006$, respectively).

Longitudinally opened descending aortas were stained en face with Oil Red O, and positively stained plaque areas were measured. Representative en face stained descending aortas from experimental groups are shown in Figure 2F. Lesion size was significantly smaller in mice immunized with a single peptide (C5aR-P1: 4.7%±0.2%, $P=0.015$; C5aR-P2: 6.1%±0.2%, $P=0.045$) or with the combined peptides (C5aR-P1+P2: 2.7%±0.4%, $P=0.008$) compared with that in control mice (9.9%±1.5%) (Figure 2G). Reduction in lesion size was expressed as a percentage of the decrease in the lesion size versus total size in the descending aorta of peptide-immunized mice compared with that in control mice (set as 0% of reduction), showing 52.5% after immunization with C5aR-P1, 38.4% with C5aR-P2, and 72.8% with the combined peptide (Figure 2G). The increased reduction in lesion size in the descending aorta from the mice immunized with the combined peptide was significant compared with that in mice immunized with either C5aR-P1 ($P<0.01$) or C5aR-P2 ($P<0.01$).

Inflammatory Cell and Foxp3-Expressing $CD4^+$ Cell Content in Lesions, Foxp3 mRNA Expression in Lymph Nodes

The percentage of macrophage-occupied area in the lesions decreased significantly. The reduction was 68.7% (10.6% versus 33.9%; $P<0.001$) in mice immunized with C5aR-P1, 63.7% (12.3% versus 33.9%; $P<0.001$) in mice immunized with C5aR-P2, and 82.1% (6.1% versus 33.9%; $P<0.001$) in mice immunized with C5aR-P1+C5aR-P2 (Figure 3A and 3B). Total macrophage-occupied area in lesions in control mice (33.9%) was set at 100% (0% reduction).

The percentage of the $CD11c^+$-stained area in the lesion showed a significant reduction in mice, of 74.1% (6.9% versus 26.6%; $P<0.001$) in mice immunized with C5aR-P1, 70.0% (8.0% versus 26.6%; $P<0.001$) in mice immunized with C5aR-P2, and 81.6% (4.9% versus 26.6%; $P<0.001$) in mice immunized with C5aR-P1+C5aR-P2 compared with the $CD11c^+$ content of the lesions in control group (Figure 3C).

The proportion of $CD4^+$ cells expressing Foxp3 in the lesions was ≈3- to 4-fold higher in mice immunized with C5aR-P1 (2.8%; $P<0.001$), C5aR-P2 (2.6%; $P<0.05$), or C5aR-P1+C5aR-P2 (3.7%; $P<0.001$) compared with 0.9% in control mice (Figure 3D and 3E). In addition, higher levels of $Foxp3^+$ cells were found for the combined peptides versus those either in mice immunized with C5aR-P1 ($P<0.05$) or C5aR-P2 ($P<0.01$). The flow cytometric analysis of spleen cells showed significantly increased expression of $CD4^+CD25^+Foxp3^+$ in mice immunized with 2 peptides either alone or in combination compared with the KLH control (Figure 3F and 3G). Similar to the observation in the lesions, higher expression of $Foxp3^+$ was also found for the combined peptides versus those in either C5aR-P1 ($P<0.05$) or C5aR-P2 ($P<0.05$).

Consistently, mRNA expression of Foxp3 in lymph nodes showed 1.8-fold, 1.7-fold, and 2.2-fold increases (Figure IIIA in the online-only Data Supplement) in mice immunized with C5aR-P1, C5aR-P2, and C5aR-P1+P2, respectively, when compared with controls.

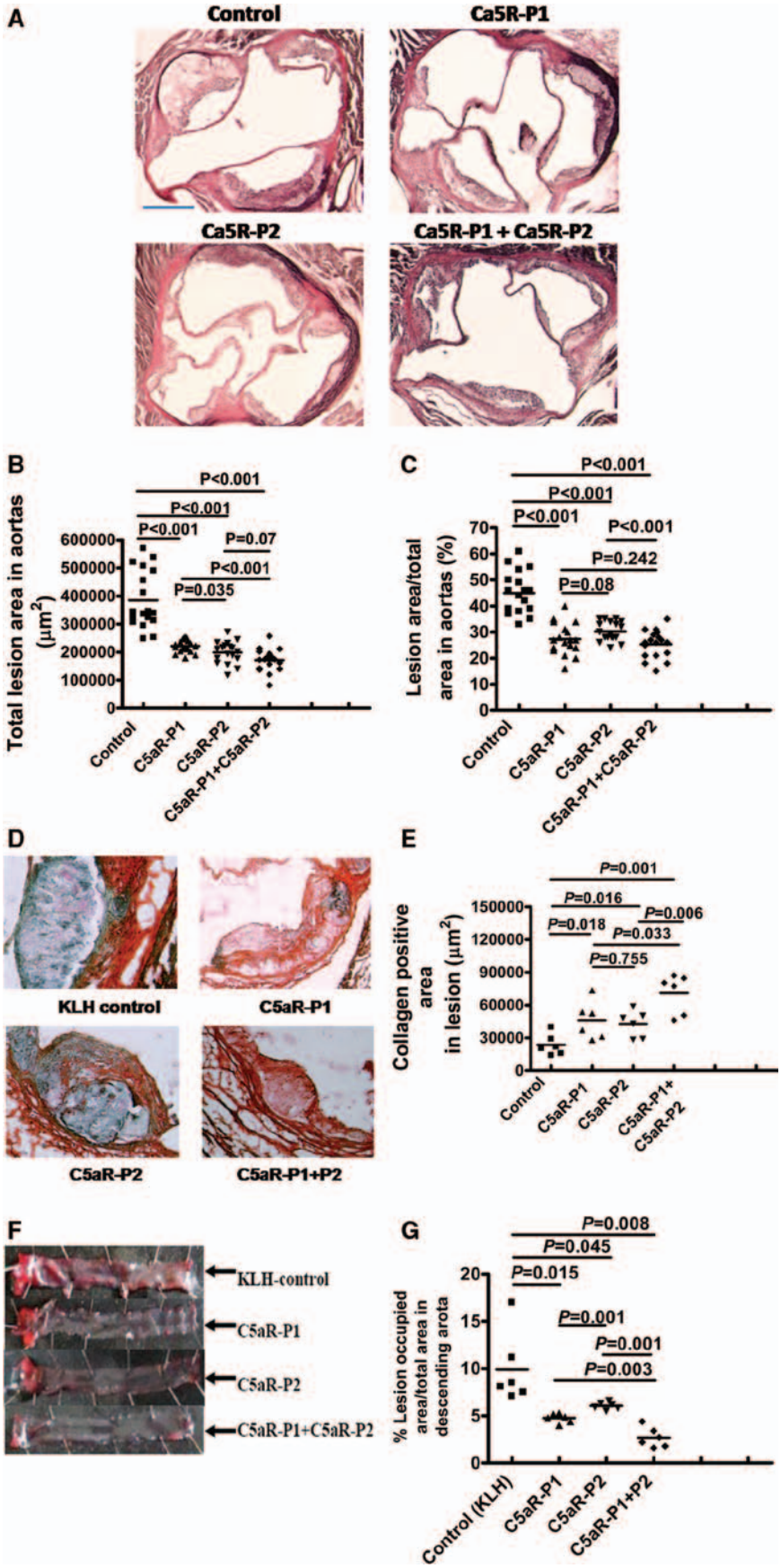

Figure 2. Detection and quantitation of lesion areas in the aorta and descending aorta of *Ldlr*^{tm1Her}*Apob*^{tm2Sgy}J mice fed a high-fat diet after immunization with C5aR peptides vs controls (keyhole limpet hemocyanin [KLH]). **A**, Representative photomicrograph of lesions observed in atherosclerotic aortas as analyzed with elastin/van Gieson staining. Scale bar: 250 μm. **B**, Lesion area in μm^2. **C**, Percentage of luminal surface occupied by lesions in the aortic sinus (percentage of ratio of lesion areas in μm^2 vs. total areas in μm^2) (n=18 sections, 3 sections per mouse). **D** and **E**, Representative photomicrographs and quantitative analysis of collagen (Sirius Red coloration under polarized light) in atherosclerotic aortas in individual mice (n=6). **F**, Representative stained en face descending aorta from mice. **G**, Percentage of lesion-occupied area vs total area of descending aortas in individual mice (n=6) of the different experimental groups. The mean lesion size and the difference in lesion size between the experimental groups are shown.

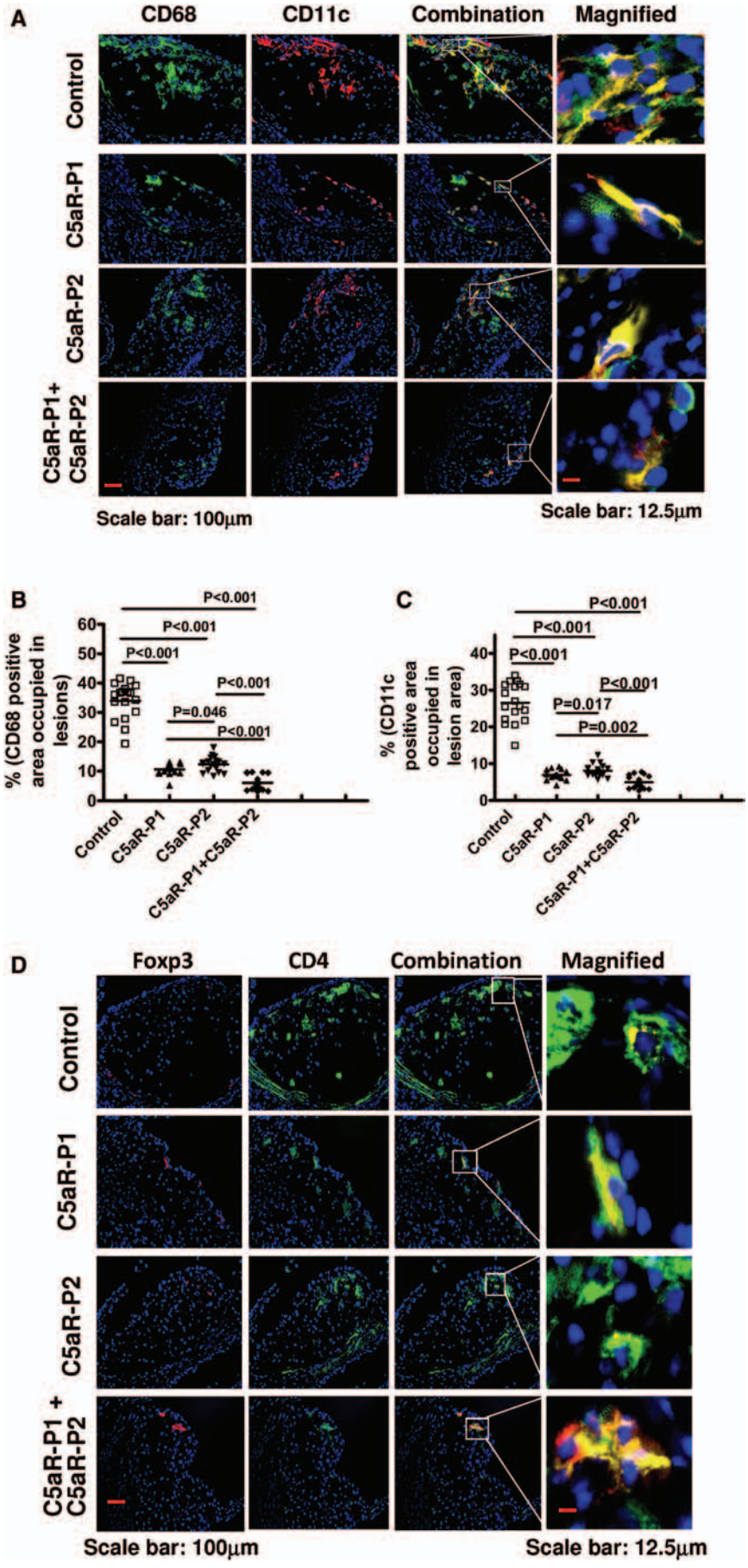

Figure 3. (Continued)

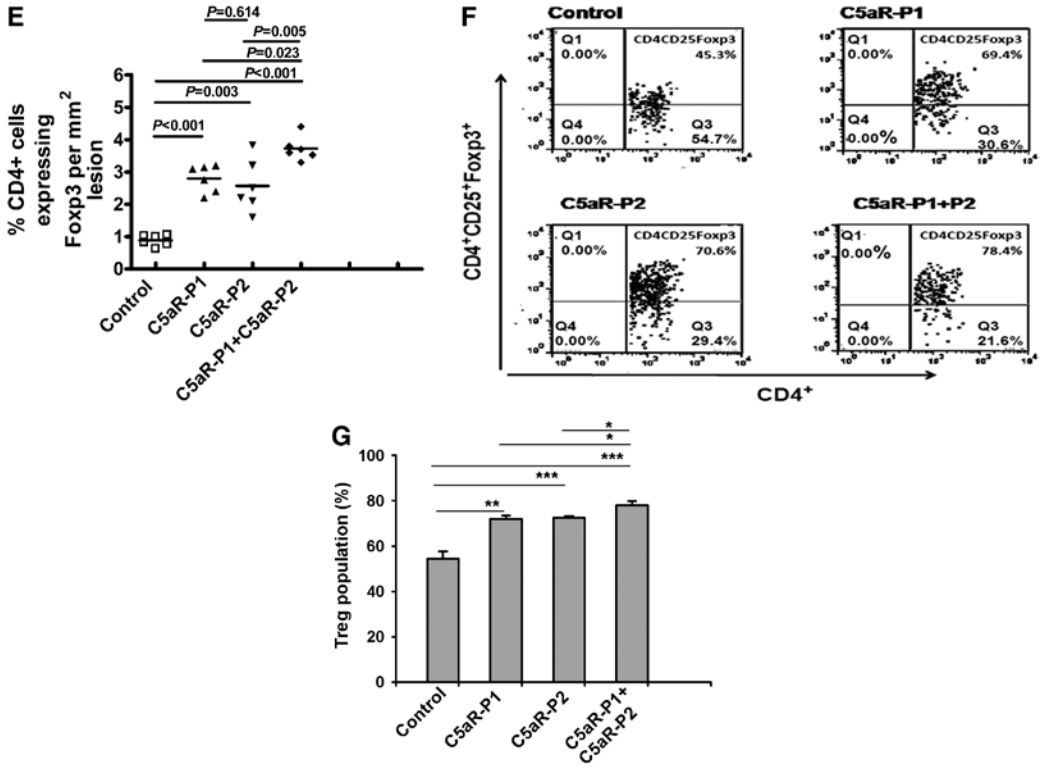

Figure 3. Assessment of the presence of inflammation-associated cells in lesions of *Ldlr*^{tm1Her}*Apob*^{tm2Sgy}J mice fed a high-fat diet after immunization with C5aR-P1, C5aR-P2, or C5aR-P1+C5aR-P2. **A**, Representative photomicrographs showing immunohistochemical staining of CD68 (green) and CD11c (red) markers in the aorta sections of different experiment groups. **B**, Percentage of positively stained lesion areas vs total lesion area for CD68. **C**, Percentage of positively stained lesion areas vs total lesion area for CD11c. **D**, Photomicrographs showing immunohistochemical staining of CD4$^+$ T cells (green) and Foxp3$^+$ Treg cells (red). **E**, Assessment of Treg cells as percentage of Foxp3$^+$ areas colocalized with CD4$^+$ area shown in yellow (n=18 sections). **F**, Representative flow cytometric plots for CD4$^+$CD25$^+$Foxp3$^+$ (Treg) cell population in spleen cells. Spleen cells from mice immunized with either C5aR peptides or keyhole limpet hemocyanin (KLH) (control) were performed using a Treg detection kit (Miltenyi Biotec, Surrey, UK) according to manufacturer's protocols. **G,** Bar chart presentation of flow cytometric analysis. Data represent mean±SEM from 3 independent samples. **P*<0.05, ***P*<0.01, and ****P*<0.001. Foxp3 indicates forkhead box protein-3.

Expression of Anti- and Proinflammatory Cytokines in Lesion Sites, mRNA Expression of Cytokine in Aorta Arch, Cytokine Concentrations in Plasma and in Supernatants of Stimulated Splenocytes

Anti-inflammatory cytokine IL-10 expression, as detected by immunohistochemistry, in the aortic lesions of mice immunized with the peptides is shown in Figure 4A. The proportion of CD4$^+$ cells expressing IL-10 per mm^2 lesion was ≈1.5-fold higher in mice immunized with the combined peptides compared with peptides used alone (3.3%±0.4% versus 4.5%±0.4% for C5aR-P1; 3.2%±0.4% for C5aR-P2; P<0.05). The higher IL-10 content was also found for peptides used alone versus those in controls (0.9%±0.1%) (Figure 4B).

Immunohistochemical analyses showed significantly smaller TNF-α occupied areas in the lesions of mice immunized with the combination peptides compared with peptides used alone (6.7%±0.47% for C5aR-P1+C5aR-P2 versus 17.2%±1.0% for C5aR-P1 and 14.0%±1.5% for C5aR-P2). In addition, a reduction in the TNF-α–occupied lesion areas was observed in mice receiving peptides alone compared with controls (44.5%±2.4%) (Figure 4C and 4D).

Consistently, mRNA expression of cytokines in the aorta arch showed 3.8-fold, 1.3-fold, and 5.1-fold increases in the expression of IL-10 (Figure IIIB and IIIF in the online-only Data Supplement) for C5aR-P1, C5aR-P2, and C5aR-P1+P2, respectively, whereas 6.6-fold, 2.3-fold, and 16.3-fold increases in the expression of TGF–β (Figure IIIC and IIIF in the online-only Data Supplement) for C5aR-P1, C5aR-P2, and C5aR-P1+P2, respectively. By contrast, 2.6-fold, 4.5-fold, and 4.7-fold decreases were observed in the expression of TNF-α Figure IIID and IIIF in the online-only Data Supplement), and 1.1-fold, 1.4-fold, and 3.0-fold decreases in the expression of IFN-γ (Figure IIIE and IIIF in the online-only Data Supplement) for C5aR-P1, C5aR-P2, and C5aR-P1+P2, respectively. These results were consistent with cytokine levels measured in the plasma and in the supernatants of splenocytes.

Plasma concentrations of IL-10 (P<0.05) and TGF-β (P<0.01) were increased significantly in mice immunized with either the C5aR-P1 or C5aR-P2 peptide antigens compared with controls (Figure 4E and 4F); even higher concentrations were observed in mice immunized with the combined peptides than with either alone. The difference is significant for IL-10 (P<0.05; C5aR-P1+C5aR-P2 versus C5aR-P1) and TGF-β (P<0.05; C5aR-P1+C5aR-P2 versus either C5aR-P1 or C5aR-P2). In contrast, the plasma concentration

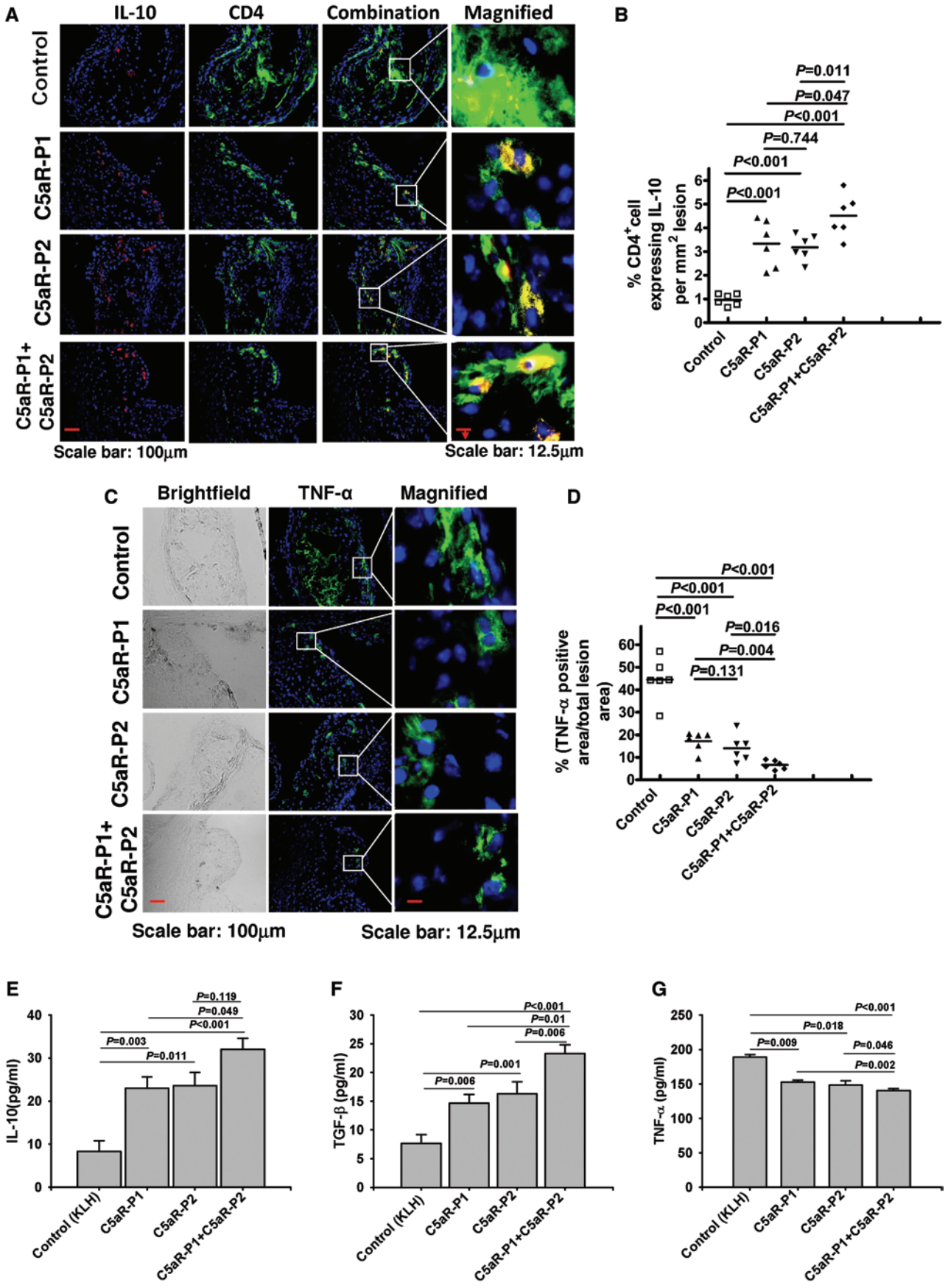

Figure 4. (Continued)

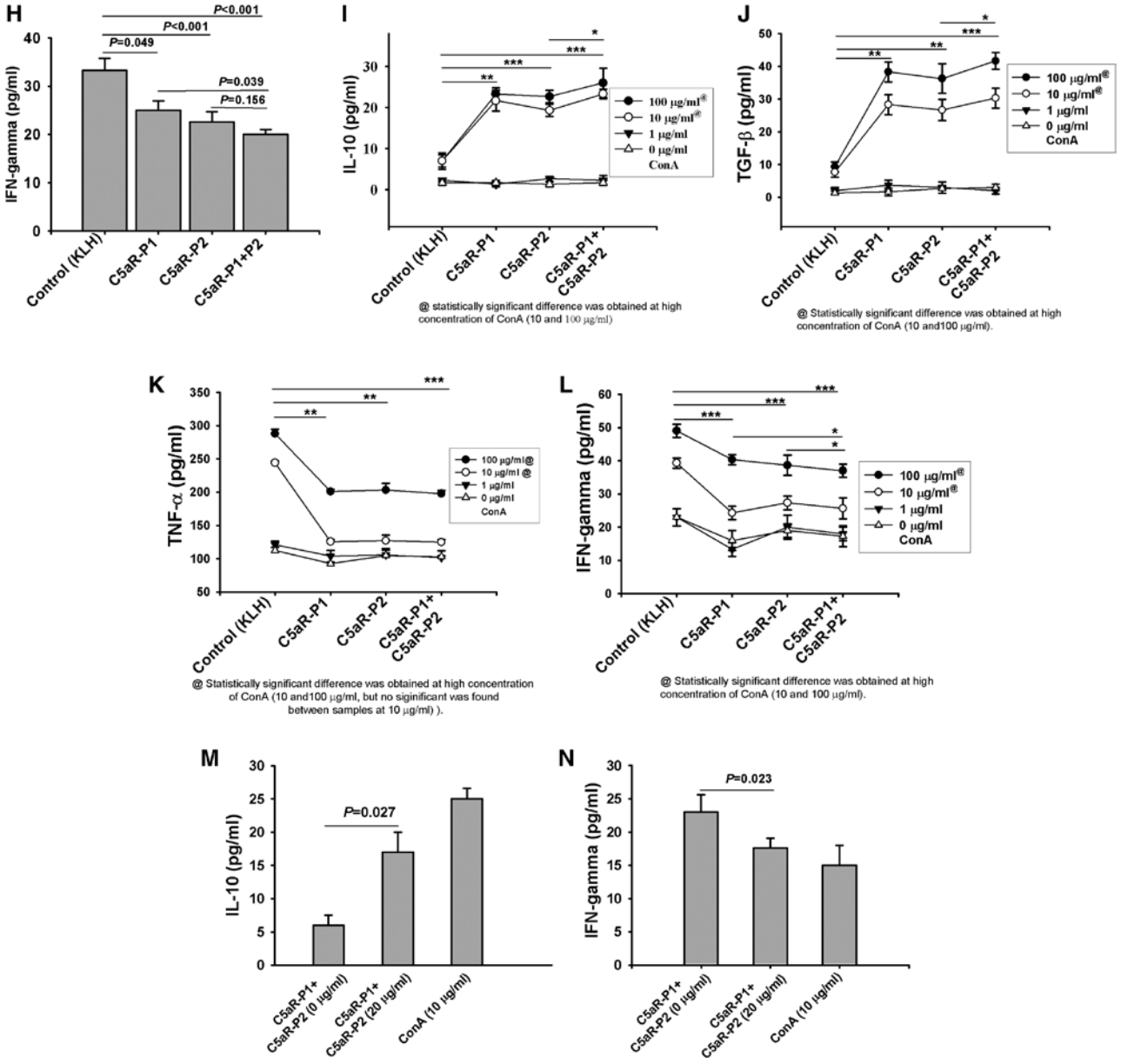

Figure 4. Assessment of cytokine concentrations in lesion sites, plasma, and supernatant of spleen cells stimulated by either ConA or C5aR peptides. **A**, Representative photomicrographs showing dual-immunohistochemical staining of aorta section of mice from different experimental groups for interleukin (IL)-10-producing T cells, IL-10 (red), and CD4 (green). **B**, Percentage of $CD4^+$ cell expressing IL-10 per mm^2 lesion. The figures are based on values from all animals using the mean of 3 sections from each animal (n=6 mice). **C**, Representative photomicrographs for tumor necrosis factor (TNF)-α-positive area (green) in aorta sections from mice of different experimental groups. **D**, Percentage of TNF-α-positive area of the lesions vs total lesion area (n=18 sections). Magnification is same as that in (4**A**).
E–H, Concentrations of cytokines IL-10, transforming growth factor (TGF)-β, TNF-α, and interferon (IFN)-γ in plasma from *Ldlr*^{tm1Her}*Apob*^{tm2Sgy}J mice fed a high-fat diet after immunization with peptide antigens or with control keyhole limpet hemocyanin (KLH). **I–L**, Concentrations of cytokines for IL-10, TGF-β, TNF-α, and IFN-γ in the supernatant of splenocytes stimulated with different concentrations of ConA from *Ldlr*^{tm1Her}*Apob*^{tm2Sgy}J mice fed a high-fat diet after immunization with peptide antigens or with control KLH. **M** and **N**, Concentration of cytokine for IL-10 and IFN-γ in the supernatant of splenocytes stimulated with C5aR-P1+C5aR-P2 (10 µg/mL for each peptide) from *Ldlr*^{tm1Her}*Apob*^{tm2Sgy}J mice fed a high-fat diet after immunization with C5aR-P1+C5aR-P2 (5 analyses±SEM and differences between experimental groups are shown). Data represent mean±SEM from 6 mice of each group. **P*<0.05, ***P*<0.01, and ****P*<0.001.

of the atherogenic cytokine TNF-α was reduced (*P*<0.05) by immunization with the 2 peptide antigens individually compared with that in controls, whereas a significant reduction of TNF-α was observed in mice immunized with C5aR-P1+C5aR-P2 versus controls (*P*<0.001) (Figure 4G). A significant difference was also shown in mice immunized with the peptides given in combination versus individually. A trend similar to that observed for the plasma concentrations of TNF-α was found with respect to the plasma levels of IFN-γ after immunization with the peptides used either alone or in combination (Figure 4H). Although no statistically significant difference was found between mice immunized with C5aR-P2 and C5aR-P1+C5aR-P2 (*P*=0.156) in plasma levels of IFN-γ, a significant difference was found between mice

immunized with C5aR-P1 and C5aR-P1+C5aR-P2 (P=0.039) (Figure 4H).

Supernatants of splenocytes from mice immunized with these peptide antigens used either alone or in combination showed significantly increased secretions of IL-10 (Figure 4I) and TGF-β (Figure 4J), when stimulated with 10 or 100 μg/mL of ConA (P<0.05–0.001). The secretion of IL-10 in C5aR-P1+C5aR-P2-immunized mice was significantly higher than for C5aR-P2 used alone. In contrast, the decreased secretion of TNF-α (Figure 4K) and IFN-γ (Figure 4L) was found in supernatants of splenocytes in mice immunized with these 2 peptides used either alone or in combination (P<0.01–0.001) when compared with controls. Again, a significantly greater decrease in the secretion of IFN-γ was observed in mice immunized with peptides used in combination versus alone (Figure 4K; P<0.05–0.01), but this was not the case with respect to the secretion of TNF-α, which showed no difference between peptides used either alone or in combination. IL-10 and IFN-γ produced by splenocytes from the mice immunized with a combination of C5aR peptides after 48-hour incubation with the same peptides were measured as well. Incubation with both C5aR-P1 and -P2 peptides resulted in a significantly larger production of IL-10 (17±3.0 versus 6±1.5; P=0.027) and a significantly decreased production of IFN-γ (23±2.6 versus 18.6±1.5; P=0.023) when compared with the spleen cells cultured without C5aR peptides (Figure 4M and 4N; the ConA used as a positive control).

Antigen-Specific Regulation Function

$CD4^+$ T cells from the spleens of mice immunized with the peptide antigens were incubated with the corresponding immunizing antigens at 0–10 μmol/L, and a dose-dependent significant increase in proliferative response was shown between 0.1 and 1 μmol/L of peptide concentrations (Figure IV in the online-only Data Supplement). $CD4^+$ T-cell proliferative responses were observed from antigen-immunized mice incubated with the corresponding immunizing antigen and the response was specific (Figures IVA–IVC in the online-only Data Supplement) compared with those incubated with KLH control in which the responses were smaller and nonspecific (Figure IVD in the online-only Data Supplement). Because the proliferative response can only be used as a general indicator of T-cell reactivity, we further assessed whether functional Treg cells were induced by immunization. We cocultured antigen-specific (for the C5aR epitope) Treg cells with $CD4^+$ effector T cells ($CD4^+CD25^-$ T cells). T-effector-cell proliferation was suppressed in a dose-dependent manner in the presence of Treg cells from C5aR peptide–immunized mice either alone or in combination (Figure 5A–5C). The differences were significant when adding equal or half the amount of Tregs into the effector cells (1:1 and 2:1) (P<0.01), with the exception of 4:1 in mice immunized with both peptides simultaneously (P<0.05). No such suppression was observed from KLH-immunized control mice (Figure 5D). In addition, Treg cells from KLH-control mice did not suppress T-effector-cell proliferation in the presence of C5aR peptides, further supporting the antigen specificity of the Treg-cell function.

Evaluation of Monocyte Differentiation Into Macrophages in PBMC From C57BL/6 Background Naïve Mice and Detection of Spleen Cells From Peptide-Immunized or KLH-Immunized Mice

Mouse (C57BL/6) PBMCs were stimulated with granulocyte-macrophage colony-stimulating factor in the presence or absence of C5a or C5a antisera/C5aR peptide antisera.

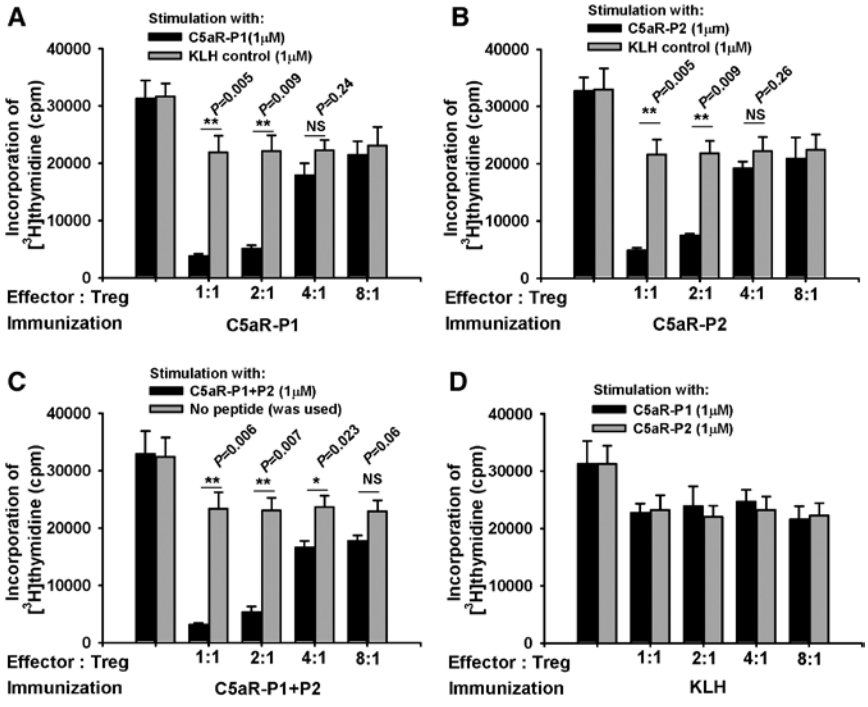

Figure 5. Assessment of antigen-specific regulatory function. T-effector cells, the $CD4^+CD25^-$ cells, were isolated from $CD4^+$ T cells (unbound to $CD4^+CD25^+$ cell beads, 99.5% of $CD4^+CD25^-$ cells) from spleen of mice that had received oral immunization with C5aR-P1, C5aR-P2, and C5aR-P1+P2, respectively. Proliferation of effector cells alone is indicated in the leftmost bar of each group. Addition of isolated $CD4^+CD25^+$ Treg cells from animals immunized (**A**) with C5aR-P1, (**B**) with C5aR-P2, (**C**) with C5aR-P1+ P2, and (**D**) with keyhole limpet hemocyanin (KLH) control is indicated at different ratios to affect cells. Data are expressed as mean of 6 analyses±SEM. Differences between groups are shown; *P<0.05 **P<0.01, and ***P<0.001.

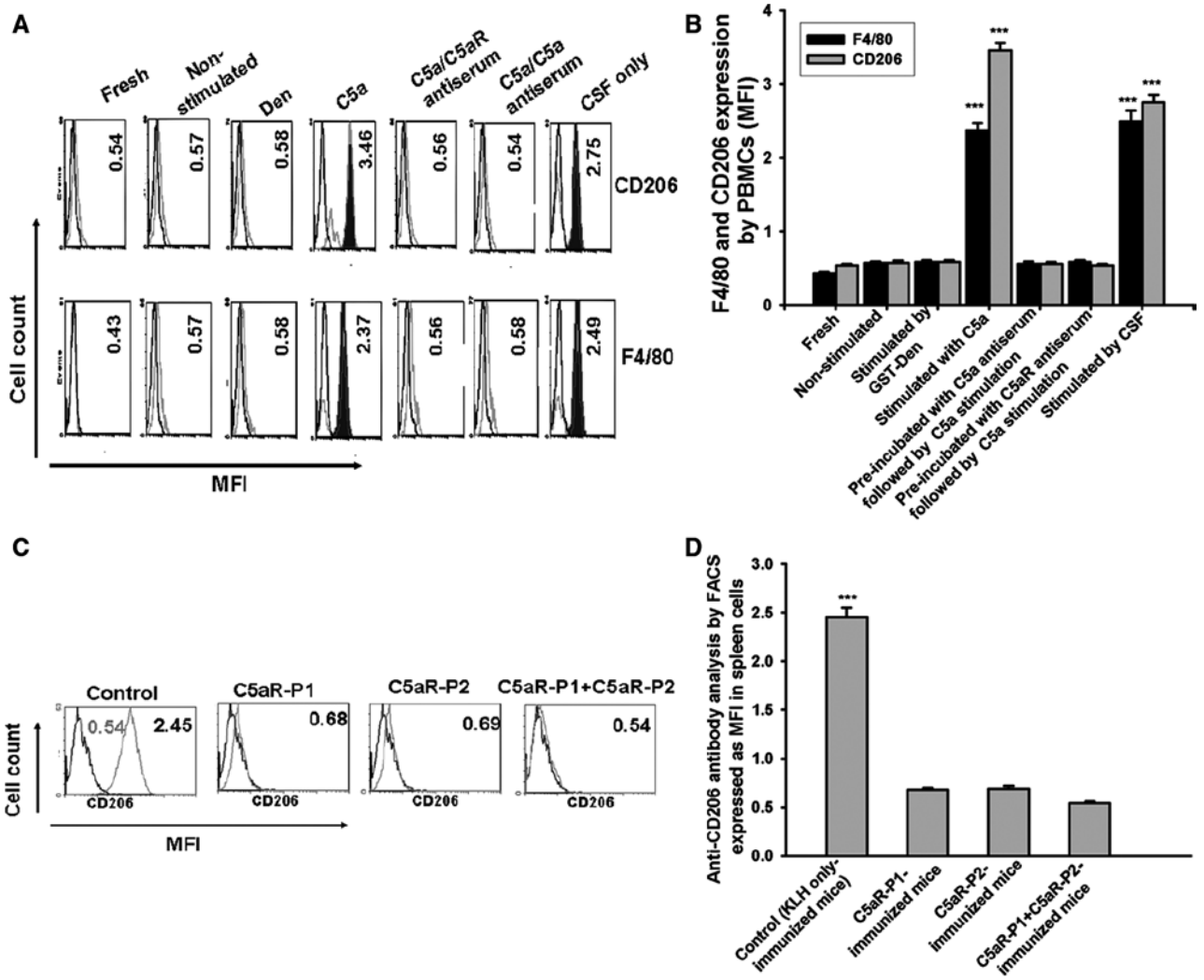

Figure 6. Measurement of monocyte differentiation into macrophages by flow cytometry. **A**, Peripheral blood mononuclear cells (PBMCs) isolated from C57/B6 background mice (same background as *Ldlr*^{tm1Her}*Apob*^{tm2Sgy}J mice) were cultured for 3 days and their differentiations determined by assessing expression of CD206 and F4/80 using flow cytometry. Histograms show expressions in fresh cells (control), unstimulated cells (control), dendroaspin-stimulated (10 ng/mL) cells, C5a-stimulated (10 ng/mL) cells, C5a/C5aR antiserum (C5a stimulation after C5aR antiserum-pretreatment) treated cells, C5a/C5a antiserum (C5a stimulation after C5a-antiserum pretreatment) treated cells, and granulocyte-macrophage colony-stimulating factor-(GM-CSF) stimulated cells (shown in red) compared with that of isotype control (shown in black). Numbers show mean fluorescence intensity (MFI). **B**, CD206 and F4/80 expression in peripheral blood mononuclear cells (PBMCs) of C57/B6 mice presented as MFI. **C**, Spleen cells from C5aR-peptide-immunized mice were analyzed using a flow cytometer, and expression of CD206 was detected. Histogram presentations for CD206 expression by spleen cells of mice in different groups and numbers showing MFI. **D**, MFI values for CD206 expression by splenocytes. Data represent mean±SEM from 3 independent samples. ***P<0.001.

After 3 days, the expression of cell-surface markers CD206 (macrophage marker) and F4/80 (monocyte/macrophage marker) was assessed. C5a induced monocyte differentiation into macrophages (based on the cell number changes) when compared with fresh cells or nonstimulated cells or stimulated with dendroaspin (unrelated protein), in which case little differentiation was observed. However, PBMC differentiation induced by C5a was abolished by preincubation of the cells with the antiserum of either C5a or C5aR (P<0.001; Figure 6A and 6B). Concordantly, fewer macrophages were observed in the spleens of mice immunized with C5aR peptides compared with mice immunized with KLH (control) as shown by the considerable decrease in CD206 (macrophage marker) expression on splenocytes of C5aR peptide-immunized mice when compared with that from control mice (P<0.001; Figure 6C and 6D).

Discussion

Previous studies have reported that atheroprotective effects can be achieved by subcutaneous and nasal immunization of autoantigen such as human ApoB-100 and its peptide, which was involved in antigen-specific Treg cells in mediating the autoantigen-induced immunity toward atheroprotective effects.[21–23] In this study, we investigated whether subcutaneously immunizing *Ldlr*^{tm1Her}*Apob*^{tm2Sgy}J mice with 2 peptides derived from nonautoantigen C5aR had an effect on atherosclerotic lesions when the mice were fed a high-fat diet. These 2 peptides, located in the N-terminal extracellular domain

of the receptor, were selected because they are thought to interact with C5a.[14] Their combination showed a synergistic effect on atherosclerotic lesion reduction, indicating that the N-terminus of C5aR aa sequence (1–35) is immunogenic and is involved in reduction of lesion through immunization in this mouse model.

We first measured whether C5a or C5aR is present in the atherosclerotic lesions. Both were found to be involved at the lesion sites, reaching approximately similar levels in experimental groups with reduced lesion development in response to immunization with C5aR peptides, alone or in combination, as well as in control groups. These findings suggest that C5a and C5aR are associated with lesion development. Surprisingly, we found that immunization with C5aR peptides obviously reduced lesion development despite that the peptides had no effect on the expression of both C5a and C5aR, suggesting that immunization with C5aR peptides may affect the function of inflammatory cells rather than the expression of C5aR and C5a. It is interesting to note by our observation that the C5a cannot effectively bind to C5aR expressed on inflammatory cells in lesions in mice immunized with C5aR peptides, indicating a possible mechanism that C5aR antibody produced by immunization with C5aR peptides may occupy the site which was for C5a binding on the surface of $C5aR^+$ cells in lesions, therefore blocking C5a binding to C5aR.

We then characterized the effect of immunization with 2 C5aR peptides, either alone or in combination, on the development of atherosclerotic lesions. The results from our study show that immunization induced an IgG1 isotype specific to each antigen peptide, alone or in combination, when an individual peptide was used as an ELISA antigen. However, these peptides could induce only a weak peptide-specific IgG2c isotype response based on the observed optical density values, suggesting that vaccination with these peptides acts predominantly through the Th2 pathway. Interestingly, the anti-oxLDL and anti-native LDL IgG and IgM were detected in mice immunized with 2 peptides and fed normal chow, suggesting that cross-reactivity exists in C5aR-peptide-immunized mice. The reason we used normal chow was in view of the fact that a high-fat diet may affect the levels of anti-oxLDL and anti-native LDL IgG and IgM.[24] Indeed, several locations in the ApoB (a protein of oxLDL) sequence have a high percentage of homology to the sequence of either C5aR-P1 or C5aR-P2, but this is not the case for ApoB peptide (aa 688–707), which shows little homology to C5aR peptides using the computer-based software SIM-Alignment Tool for protein or peptide sequences (http://web.expasy.org/tools/sim) (data not shown). In this regard, the former could explain why sequence homology dictates cross-reaction; the latter may be due to antigenic surfaces rather than sequence homology that controls cross-reactivity.[25] Although the precise role of anti-oxLDL IgG in atherosclerosis remains unclear, anti-oxLDL-IgM induced by C5aR peptide immunization presumably plays an atheroprotective role in this regard; Cesena et al[26] recently reported that polyclonal IgM reduces advanced spontaneous aortic atherosclerosis as well as injury-induced accelerated carotid atherosclerosis in an *ApoE*$^{-/-}$ mouse model study. Interestingly, in the present study, C5a levels in the plasma and C5aR expression in the lymph nodes in C5aR-peptide-immunized mice remained the same, but the functions (macrophage activation and inflammatory cytokine production) requiring receptor ligand interaction were impaired after immunization of C5aR peptides.

The peptide-induced immune response was associated with an antiatherogenic effect, detected as a significant reduction in the size of the atheromatous lesion. Although the type of antibody response against an antigen coexisting with expanded Treg cells specific to the same antigen remains unclear, our data clearly show predominance of a Th2 type IgG1 response, which cross-reacts with oxLDL. The induced antibodies cross-reacted with ApoB, and this self-antigen ApoB-specific humoral immune response may be associated with a cellular response.[9]

It has previously been reported that decreased collagen content and increased concentrations of macrophages, activated $CD4^+$ T cells, and dendritic cells (markers of early lesion formation) occur during lesion formation.[21,27–29] In that perspective, it is interesting that our data demonstrate that immunization influences collagen content in lesions and cellular infiltration into atherosclerotic lesions because relatively increased collagen content, low numbers of macrophages, $CD4^+$ T cells, and $CD11c^+$ cells, and high numbers of Treg cells were observed in lesions in mice immunized with these peptides.

The additive effect of immunization with a combination of peptides to that achieved by immunization with each alone suggests that the binding site on the C5aR N-terminus overlaps with the 2 peptides, although it cannot be excluded that certain residues at the N-terminus of C5aR-P1 or the C-terminus of C5aR-P2 are not involved in reduction of lesion.

TNF-α and IFN-γ are crucially involved in the progression of atherosclerosis.[30,31] On the basis of the cytokine profiles from either plasma or spleen cells in immunized mice, TNF-α and IFN-γ secretion may well be linked to the ability of the immune animals to release these cytokines from preprimed cells, leading to a decrease in the need to synthesize significant amounts of new TNF-α and IFN-γ. Our results show that vaccination with peptides promoted a major shift away from proinflammatory cytokines (ie, TNF-α and IFN-γ) toward anti-inflammatory cytokines (IL-10 and TGF-β), which is evident not only in the plaque but also systemically. These results were also consistent with the up-regulating anti-inflammatory cytokine mRNA expression and down-regulating atherogenic cytokine mRNA expression. Although these results strongly suggest that an anti-inflammatory response is responsible for the observed reduction in plaque size, they do not discriminate the reductions attributed to either of the anti-inflammatory cytokines, as both IL-10 and TGF-β have been implicated in playing a role in atherosclerosis.[32,33] A strategy using TGF-β knockout mice is planned in a future study to delineate the relative contributions of these 2 cytokines.

The $CD4^+$ T-cell-specific proliferative response in the C5aR peptide-immunized mice indicated that a cellular immune response was involved in the reduction of atherosclerotic lesion size. The mechanism of this reduction may be due to C5aR peptide immunization blocking monocyte differentiation into macrophages through the regulation of the C5aR/C5a

interaction. Our findings show that using C5aR peptides to immunize mice will not affect C5aR expression and C5a release from cells but will affect the ability of inflammatory cells to secrete cytokines. One possible explanation could be that C5a could not exert its cellular reaction in C5aR-peptide-immunized mice because C5aR seemed to lose its binding site to C5a. In addition, the atheroprotective effect paralleled an induction of $CD4^+CD25^+$ Treg suppression of C5aR-peptide-specific effector T cells in vitro. Furthermore, our findings also show that Foxp3 seems to be highly expressed in aortas, splenocytes, and lymph nodes in mice immunized with C5aR peptides as $Foxp3^+$ Treg cells are known to be atheroprotective.[34] Although $CD4^+CD25^-$ T cells also expressed Foxp3, or regain CD25 expression and act as regulatory cells,[35] the degree of expression of Foxp3 was significantly less than that of $CD4^+CD25^+$ T cells, at least under the conditions described in our study, there was no evidence of a regulatory effect for purified $CD4^+CD25^-$ cells.

Antigen-specific regulation function has been demonstrated in intranasal immunization[22] and subcutaneous immunization[21,23] using the same epitope within ApoB-100. In agreement with our present findings, it appears that modulation in atherosclerosis-related autoimmunity by antigen-specific activation of Tregs represents a novel approach for the treatment of atherosclerosis. We also found that both C5a protein and C5aR are expressed in the lesion sites of aorta sinus in mice immunized with ApoB peptide or with a human heat shock protein 60 peptide, as well as with KLH control (Figure IF–IH in the online-only Data Supplement). Interestingly, colocalization of C5a with C5aR was shown at similar levels in the lesions of peptide-immunized and control mice (Figure IH in the online-only Data Supplement), suggesting that C5a and C5aR are associated with lesion development through a different mechanism from that associated with ApoB peptide or human heat shock protein 60 peptide; therefore, immunization with the C5aR peptides reducing lesion size may be through a different pathway from that associated with immunization of ApoB or human heat shock protein 60 peptides. In this regard, C5aR peptide immunization implies that C5aR could serve as a useful target for the development of antiatherosclerotic agents particularly as the binding of C5a to C5aR is known to exert a range of biological effects, which may contribute to the formation of atherosclerosis. In agreement with the findings of the present study, another recently reported study of the effect of treatment of $ApoE^{-/-}$ mice with a CD88 (C5aR) antagonist (PMX53) showed a reduction in lesion size, substantiating a role for C5a in atherosclerosis.[36] In addition, C5a and C5aR are expressed in human coronary plaque.[37,38] Conversely, a study using C5/ApoE double-knockout mice fed a high-fat diet failed to demonstrate any difference between C5-deficient and C5-sufficient animals, suggesting little contribution to atherosclerosis by C5a[39]; these results could be explained as attributable to the increased inflammatory environment overriding any effects of C5 deficiency as using a high-fat diet is associated with increased release of inflammatory cytokines and chemokines,[40,41] and of oxLDL antibody.[23]

Our data show that antibodies produced by immunization of C5aR peptides could prevent monocyte differentiation into macrophages, an important step in disease progression. Therefore, it is feasible to propose that the mechanism responsible for the ability of C5aR-derived peptide antibodies to reduce atherosclerotic lesions could be due to both a humoral response as well as a cell-mediated regulatory response to the antigen; immunization-induced specific Treg cells dampen the atherogenic ApoB-specific cellular immune response; binding of C5aR peptide-induced antibodies to oxLDL could potentially decrease proinflammatory activities, thereby reducing atherosclerosis.

The precise role of the C5a/C5aR interaction in atherosclerosis remains, however, the subject of continuing investigation. Our present study reported for the first time that C5aR N-terminal peptide immunization is able to modulate oxLDL-specific immunity (cross-reaction) and highlights that the effect of immunization of these C5aR peptide epitopes on the reduction of atherosclerotic lesion formation through the mechanisms of inducing a specific Treg-cell response as well as blockage of monocyte differentiation into macrophages.

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Disclosures

Drs Lu and Kakkar are the inventors of a patent regarding C5aR-related vaccine against atherosclerosis.

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Immunization With a Combination of 2 Peptides Derived From the C5a Receptor Significantly Reduces Early Atherosclerotic Lesion in *Ldlr*^{tm1Her} *Apob*^{tm2Sgy} J Mice

Xinjie Lu, Min Xia, Valeria Endresz, Ildiko Faludi, Lakshmi Mundkur, Eva Gonczol, Daxin Chen and Vijay V. Kakkar

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ONLINE SUPPLEMENT

Materials and Methods

Animal Experiments

Female *Ldlr*^{tm1Her} *Apob*^{tm2Sgy}J mice (The Jackson Laboratory, Maine, USA) were used, with a total of 5 groups (3 sample groups and 2 control groups), each comprising 8–10 female mice (5–6-weeks' old) with similar body weights. For subcutaneously immunization with the antigens, the repetitive immunization multiple sites strategy (RIMMS) was adopted [1,2]. Mice were inoculated 5 times at 2–3-day intervals. At each immunization mice received 20μg of KLH-conjugated peptide mixed with 50 μl Alum adjuvant (Aluminum hydroxide gel, Sigma). Group 1 received KLH–C5aR-P1 (peptide 1), group 2 received KLH–C5aR-P2 (peptide 2), group 3 received a combination of KLH–C5aR-P1 and KLH-C5aR-P2 (10 μg each), group 4 were injected with a combination of KLH and Alum, and group 5 were injected with Alum only and for the purpose of observation of immune response only. According to a modified RIMMS protocol, this mix was subcutaneously injected into 8 sites (30 μL for each site) [1,2]. For the study of cross-reaction with oxLDL and native LDL, 3 additional groups of mice (6 per group) were immunized with C5aR-P1, C5aR-P2, and control KLH. Mice were fed with normal chow for 5 weeks. For isolation of T-effector cells, additional groups of mice (8 per group) (KLH only, C5aR-P1, C5aR-P2, and C5aR-P1+C5aR-P2) were administered orally with 20 μg of antigen on days 0, 2, 5, 7 and 9 (5 times) using a gavaging needle. Mice were given 2 mg of soybean trypsin inhibitor before oral feeding. Peptides were synthesized and conjugated to KLH by Severn Biotech Ltd, UK. From the third week of the experiment the mice were fed a high-fat diet (a modified recipe of TD96121 and TD88137 with 21% anhydrous milk fat and 1.25% cholesterol (Altromin Spezialfutter GmbH & Co, Germany) for 10 weeks.

Antibody Response Measurement

Blood samples were collected in heparinized capillaries by retro-orbital bleeding at week 2, and 12 weeks after the first injection of the antigens to test antibody production. The free C5aR-P1 and C5aR-P2 containing an N-terminal cysteine (synthesized by Severn Biotech Ltd, Worcestershire, UK) were used in ELISA as antigens. Maleimide-activated 96-well plates (Pierce, Thermo Fisher Scientific Inc., Hampshire, UK) were coated with these peptides individually and peptide-specific IgG1 and IgG2c were detected in the plasma of immunized mice according to the manufacturer's instructions. 1:1250 and 1:6250 dilutions of plasma samples were prepared before assaying for peptide-specific IgG1 and 1:50 and 1:250 dilutions were used for IgG2c detection. Horseradish peroxidase (HRP)-conjugated anti-mouse IgG1 (Biosource, San Joe, CA, USA) and HRP-conjugated anti-mouse IgG2c (Abcam, Cambridge, UK) were used as the secondary antibodies.

For ELISA analysis of cross-reaction with oxLDL/LDL, microtiter plates were coated with 15 μg/mL of LDL or oxLDL in a coating buffer with 100 mM $NaHCO_3$ and 1 mg/mL Na EDTA. Plates were then washed thoroughly with PBS with 0.05% Tween-20 and incubated with PBS with 5% BSA for 30 min at room temperature to prevent nonspecific binding. Mouse sera were incubated for 1 h at 37°C in duplicate wells, using a serum dilution of 1/100 in PBS Tween. After washing, HRP-conjugated antibodies to mouse IgM/IgG were used as the secondary antibodies. Copper oxidation of LDL was performed by incubation of LDL (1 mg of protein/mL in EDTA-free PBS) with copper sulfate (10 mM) for 24 h at 37°C. Oxidation was terminated with 10 mM EDTA and dialysis against 150 mM NaCl and 0.01% EDTA to remove $CuSO_4$. We also measured cross reaction using previously stored serum from ApoB peptide (aa 688-707)-immunized mice [2] against C5aR peptides as antigens. For the measurement of C5a concentration in plasma, a mouse C5a (mC5a) ELISA kit (IBL International GmbH, Hamburg, Germany) was used according to the manufacturer's instructions. For the measurement of oxLDL concentration in plasma, a mouse oxLDL ELISA kit (USCN, life Science Inc, Wuhan, China) was used according to the manufacturer's instructions.

Tissue Preparation

Comprehensive quantification of atherosclerosis was performed in *Ldlr*^{tm1Her} *Apob*^{tm2Sgy}J mice as follows: 12 weeks after the first immunization, tissues were harvested and mounted in optimal cutting

temperature (OCT) for immunohistochemical analyses or paraffin for lesion measurement. The OCT-embedded samples were frozen in the mounting medium (OCT compound, Tissue-Tek, Sakura Finetek, Europe) from which 5-μm thick sequential sections were taken using a Reichert-Jung Cryocut 1800 (Leica). The paraffin-embedded sections (5-μm thick) were prepared using a Leica Jung RM2055 microtome.

Morphometric Analyses, Quantitative Measurements of Atherosclerosis, and Composition of Atherosclerotic Lesion

Slides with paraffin-embedded sections were stained with hematoxylin and eosin and elastin/van Gieson (Sigma) for histological evaluation using an Olympus U-ULH optical microscope (Olympus Optical Co. Ltd, Tokyo, Japan). Image-Pro Plus TM software version 4.0 (Media Cybernetics, Silver Spring, USA) was used to trace the external elastic lamina, internal elastic lamina, and lumen in the aortic root area to ascertain the area of atherosclerotic lesions. The total aortic root area and lesion area were measured following which the ratio of total lesion area/total aortic root area was calculated and expressed as percentage of lesion in the aortic root area. Collagen was detected using Sirius Red (Sigma, Dorset, UK) staining.

Immunohistochemical Analyses

Hearts with proximal aortas embedded in OCT were sectioned and fixed in methanol. Consecutive tissue sections were incubated in 0.2% TritonX-100/phosphate-buffered saline (PBS) for 1 hour then blocked with 1% bovine serum albumin (BSA) for 1 hour. The samples were stained with either purified hamster anti-mouse CD11c (eBioscience, Ltd., Hatfield, UK) or rat anti-mouse CD4 (BD Biosciences, Oxford, UK), CD68, Foxp3, or TNF-α (BioLegend, San Diego, CA, USA) at 4°C overnight, washed in PBS and incubated with rabbit anti-rat IgG-fluorescein isothiocyanate (FITC) or goat anti-mouse IgG-tetramethylrhodamine-5-(and-6)-isothiocyanate (TRITC) (Sigma-Aldrich Company Ltd. Dorset, UK). For detection of IL-10 producing $CD4^+$ T cells and for $Foxp3^+$-CD4 T-cells (BD Biosciences, UK), FITC-conjugated rat anti-mouse CD4 mAb and phycoerythrin (PE)-labeled anti-mouse IL-10 and Foxp3 (BioLegend, San Diego, CA, USA) were used. All slides were counter-stained with mounting medium containing 4, 6-diamino-2-phenylindole (DAPI) (Vector Laboratories Inc., Peterborough, UK). Bright-field images were captured, scanned, and overlaid using an Axiovert

S100 TV immunofluorescence microscope (Zeiss, Welwyn Garden City, UK) equipped with Plan-NEOFLUAR objectives and a KTL/CCD-1300/Y/HS camera (Princeton Instruments, Buckinghamshire, UK). Image-Pro Plus TM software version 4.0 was used to determine lesion area. The content of $CD4^+$ T-lymphocytes, $CD68^+$, $CD11c^+$, and $foxp3^+$ cells within the lesion were measured with a microscope using a magnification as indicated in the related figures and averaged in μm^2. Three sections from each animal (six animals from each group) were investigated. For C5a and C5aR staining, rabbit polyclonal anti-mouse C5a antibody (Abbiotec, LLC, CA92126 USA) and rat anti-mouse C5aR monoclonal antibody (Thermo, Rockford, IL USA) were used.

Plasma-lipoprotein Analysis

Plasma total cholesterol, triacylglycerol, and high-density lipoprotein (HDL) were measured using the enzymatic colorimetric method in a P800 modular chemical analyzer (Roche Diagnostics, Mannheim, Germany) through a service from the Department of Laboratory Medicine, University of Szeged, Hungary. Low-density lipoprotein (LDL) cholesterol was calculated according to the Friedewald formula:

$$\text{LDL concentration (mmol/L)} = \text{total cholesterol} - (\text{HDL} + \text{triacylglycerol} \times 0.46).$$

Measurement of Pro- and Anti-Inflammatory Cytokines

Concentrations of murine cytokines IL-10, TGF-β, TNF-α, and IFN-γ were measured in plasma using ELISA kits following the manufacturer's instructions (R&D systems, Abingdon, UK). IL-10 and TNF-α levels in the lesions were quantified by immunohistochemical analyses and the ratio of IL-10 and TNF-α positive area/total lesion area, respectively, was measured and calculated by using Image-Pro Plus TM, version 4.0.

Cytokine Production by Splenocytes After In Vitro Stimulation with ConA

Spleens were homogenized by pressing through 70-μm cell strainer and the cells recovered suspended in RPMI (denotes: Roswell Park Memorial Institute) 1640 complete medium supplemented with 10% fetal calf serum (FCS), 100-U/mL penicillin, and 100-µg/mL streptomycin. The cells were cultured for 48 h at 37°C in a humidified 5% CO_2 incubator at a density of 1×10^6 cells/0.1 mL medium/well of the 96-well plate with a serial 10-fold dilution of ConA (1–100 μg/mL) (Sigma, Dorset,

UK). Cytokine (IL-10, TGF-β, TNF-α, and IFN-γ) concentrations in the supernatant of stimulated spleen cells were tested using ELISA kits (R&D systems, Abingdon, UK) according to the manufacturer's instructions.

Antigen-specific Regulatory Function Assays

Antigen-specific regulatory function assay experiments were performed as previously described [3,4], with some modifications. Treg cells (95% purity) were selected from spleen $CD4^+$ T-cells of C5aR-P1-, C5aR-P2-, and C5aR-P1+P2-peptide-immunized mice using a $CD4^+CD25^+$ Treg isolation kit (Miltenyi Biotech), and T-effector $CD4^+CD25^-$ cells (unbound to the beads, <0.5% of $CD4^+CD25^+$ cells) were harvested from $CD4^+$ T-cells from spleens of mice that had received oral immunization with C5aR-P1-, C5aR-P2- and C5aR-P1+P2, respectively, using the same kit. $CD4^+CD25^-$ (2×10^5) were co-cultured with $CD4^+CD25^+$ cells (2×10^5) in the presence of 2×10^4 APC (3000 rad irradiated splenocytes) per well, and stimulated with 1-μM C5aR peptides**,** with KLH controls, or without C5aR peptides. After 3 days of culture, cells were pulsed with 0.5 μCi of [^{3}H]thymidine (Amersham, Buckinghamshire, UK) for the last 18 hours of culture, were harvested, and the incorporation of [^{3}H]thymidine was determined using a liquid scintillation spectroscopy method (1205 BetaplateTM, Turku, Finland).

$CD4^+$ T-Cell Proliferation and Cytokine Secretion Assay

$CD4^+$ T-cells were purified from spleen cells of *Ldlr*^{tm1Her} *Apob*^{tm2Sgy}J mice immunized with peptide antigens by using the magnetic-activated cell sorting $CD4^+$ T-cell Isolation Kit (Miltenyi Biotec, Surrey, UK) and used as responder cells. C5aR-P1 (amino acids 1–21) and C5aR-P2 (amino acids 19–31) were used as antigens, and γ-irradiated (30 Gy) spleen cells from PBS-treated *Ldlr*^{tm1Her} *Apob*^{tm2Sgy}J mice were used as controls. $CD4^+$ T-cells (3×10^5/well) were starved for 48 hours with serum-free culture medium, and then stimulated with γ-irradiated spleen cells (2×10^5/well) in the presence of antigens or unrelated antigens (0–10 μM) for 72 h at 37°C in a 5% CO_2, 95% air-humidified incubator. Cells were pulsed with 0.5 μCi of methyl-[^{3}H]-thymidine (Amersham Biosciences, Amersham, UK)/well for the last 18 hours of culture on day 5. Proliferation was measured as [^{3}H]TdR incorporation by liquid scintillation spectroscopy. Cytokine IFN-γ and IL-10 in the supernatant were measured using an ELISA kit.

Peripheral Blood Mononuclear Cell (PBMC) Isolation From C57BL/6 Background Mice

Blood taken from C57/BL6 background mice was diluted with PBS (1:4 dilution) and slowly layered over pre-prepared histopaque solution (1:1 v/v) in a centrifuge tube, followed by centrifugation (2000 rpm) at room temperature for 20 minutes to collect the mononuclear cells retained at the surface of histopaque solution. The isolated PBMCs were used for further flow cytometric analysis for their differentiation.

Flow Cytometric Analysis

Spleen cells from peptide- or KLH-immunized *Ldlr*^{tm1Her} *Apob*^{tm2Sgy}J mice were used for determination of CD206, a macrophage marker or PBMC from the naïve mice (C57/B6 back ground) for determination of CD206 as well as F4/80, a monocyte/macrophage marker (differentiation was observed by measuring the increased numbers of macrophages) by flow cytometric analysis. Briefly, 3×10^5 cells were incubated at 4°C for 30 min with appropriate dilutions of directly labeled monoclonal antibodies (allophycocyanin–anti-mouse CD206 antibody; BioLegend, Cambridge, UK) and FITC-anti-mouse F4/80 antibody (eBioscience, Hatfield, UK). After 2 washing steps with 0.02% BSA/PBS (pH 7.3), fluorescence was analyzed on a flow cytometer (Cytomics FC500; Bachman coulter, High Wycombe, UK). Six mice were used in each experimental group. For the measurement of C5aR concentration in the plasma, PE anti-mouse CD88 (C5aR) antibody (BioLegend UK Ltd, Cambridge, UK) was used according to manufacturer's protocols. For Treg detection, spleen cells from mice immunized with either C5aR peptides or KLH (control) were performed using a Treg detection kit (Miltenyi Biotec, Surrey, UK) according to manufacturer's protocols.

Real-Time Reverse Transcription Polymerase Chain Reaction (RT-PCR) Analysis

Total RNA from the aortic arch was isolated using TRIzol® reagent (Invitrogen, Carlsbad, California, USA). The quality of the RNA was determined by measuring the absorbance at 230, 260, and 280 nm using Nanodrop 1000. RT-PCR was performed with a 2-step SYBR superscript RT-PCR kit (Invitrogen) using the ABI PRISM 7500 sequence detection system (Applied Biosystems, 7500 real time PCR system). Mouse GAPDH was used to normalize sample amplification. The following mouse-specific primers were used to amplify the desired genes:

IL-10-F, 5'- GCTCTTACTGACTGGCATGAG-3'; IL-10-R, 5'-CGCAGCTCTAGGAGCATGTG-3'; TGF-β-F, 5'- TTGCTTCAGCTCCACAGAGA-3'; TGF-β-R, 5'- TGGTTGTAGAGGGCAAGGAC-3' IFN-γ-F, 5'-ATGAACGCTACACACTGCATC-3'; IFN-γ-R, 5'-CCATCCTTTTGCCAGTTCCTC-3' TNF-α-F, 5'-ATGAGCACAGAAAGCATGATC-3'; TNF-α-R, 5'-TACAGGCTTGTCACTCGAATT-3' GAPDH-F, 5' AACTTTGGCATTGTGGAAGG-3'; GAPDH-R, 5'-ACACATTGGGGGTAGGAACA-3'

For Foxp3 gene expression analysis in lymph nodes, the primers were used to amplify the Foxp3 gene: Foxp3-F, CCCATCCCCAGGAGTCTTG-3'; Foxp3-R, ACCATGACTAGGGGCACTGTA-3'

Data were analyzed on the basis of the relative expression method with the formula:

Fold change (normalized) = $(E_{P\text{-target}})^{\Delta CT\text{-target}}$ / $(E_{P\text{-housekeeping}})^{\Delta CT\ \text{-housekeeping}}$

E_P refers to efficiency as defined by Pfaffl [5] and is determined for the primer set that amplifies the target sequence ($E_{P\text{-target}}$) and the primer set that amplifies the housekeeping sequence ($E_{P\text{-housekeeping}}$). The term $\Delta C_{T\text{-target}}$ denotes the difference in C_T values between the control and treatment ($C_{T\text{-control}} - C_{T\text{-treatment}}$) for the target sequence and the term $\Delta C_{T\text{ -housekeeping}}$ refers to the difference in C_T values between the control and treatment ($C_{T\text{ control}} - C_{T\text{ treatment}}$) for the housekeeping sequence.

Statistical Analyses

Data are reported as mean±standard error of the mean (±SEM), unless otherwise indicated. Figures were plotted using graph-pad Prism 5.01 and Sigma plot 9.0. For atherosclerotic lesion size, data were compared and intergroup differences were conducted using one-way ANOVA for multiple comparisons and post hoc bonferroni test. Others data were analyzed using Student's *t*-test (2-tailed analyses). Non-parametric distributions were analysized using Mann-Whitney *U* test for paiwise comparisons and the Kruskal-Wallis test for multiple comparisons. Differences between groups were considered significant at P values below 0.05.

Supplementary Figure Legends

Figure I. (A) Representative photomicrographs showing immunohistochemical staining of the aortic root showed $C5aR^+$ cells (red) and $C5a^+$ area (green) in the lesion, and $C5aR^+$ cells co-localized within $C5a^+$ (yellow) in lesions from C5aR-peptide-immunized mice and KLH-immunized mice. (B) and (C) Quantitative analysis of either C5aR or C5a expression in lesions. (D) The number of $C5aR^+C5a^+$ cells and $C5aR^+C5a^-$ cells. (E) The percentage of $C5aR^+C5a^+$ cells and $C5aR^+C5a^-$ cells. (F-H) Assessment of C5a and C5aR distribution in the lesions of *Ldlr*^{tm1Her} *Apob*^{tm2Sgy}J mice fed a high-fat diet after immunization with an ApoB peptide, human heat-shock protein (HHSP)60 peptide, and a combination of ApoB and HHSP60 peptides in KLH-conjugated form, compared with KLH control (reference 2). (F) Representative photomicrographs showing immunohistochemical staining of C5aR (in red), C5a (in green), and merged C5a/C5aR. (G) C5aR-positive area in lesion. (H) Percent of C5aR-occupied lesion. (n=6 mice)

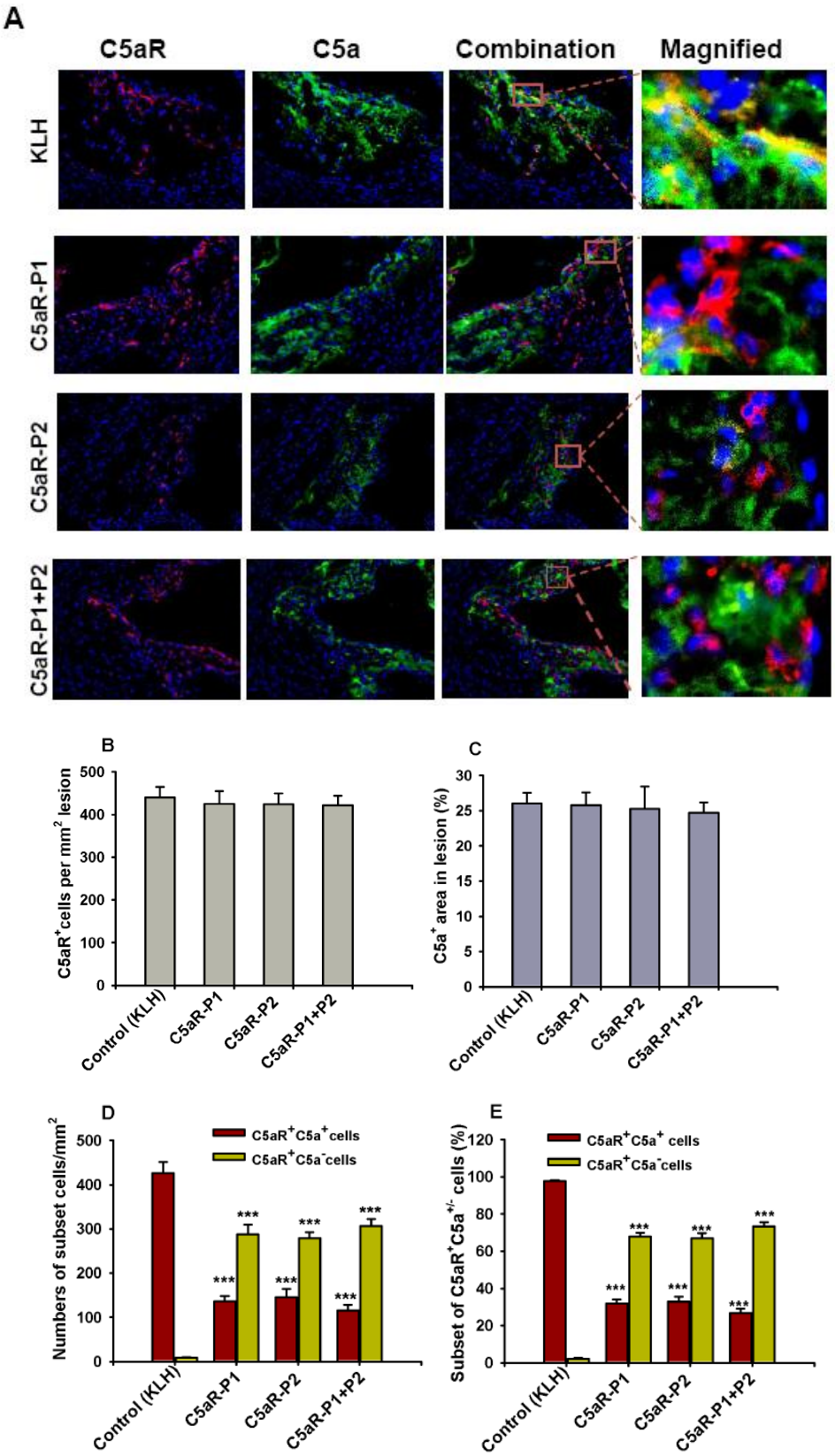
Figure II. (A) Measurement of cross-reaction using C5aR-P1- or C5aR-P2-immunized serum to react with oxLDL and native LDL. The immunized mice used were fed with normal chow for 5 weeks. (B) Measurement of cross-reaction using pooled ApoB-peptide-immunized mice serum to react with C5aR peptides. Blood samples were taken from the immunized mice at 4 weeks after the first injection of the antigens to test cross-reaction. (C) C5a concentrations in plasma observed by ELISA analysis (n=6 mice). (D) OxLDL concentrations in plasma observed by ELISA analysis (n=5 mice). (E) C5aR expression in lymph nodes analyzed by flow cytometer.

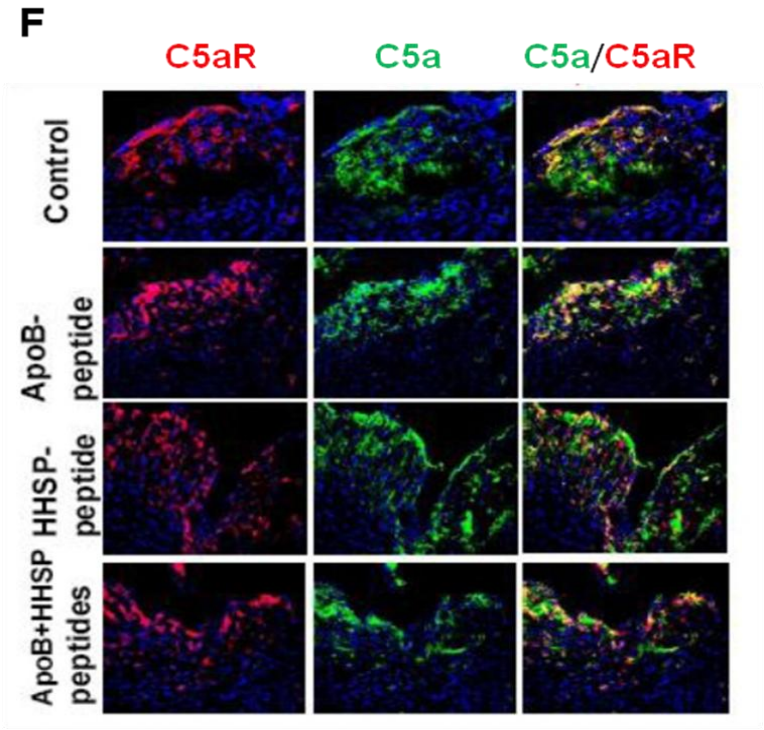
Figure III. Cytokine mRNA expression in aorta arch and Foxp3 mRNA expression in lymph nodes, respectively; Real-time reverse-transcription PCR normalized to GAPDH in aorta arches and lymph nodes of peptide-immunized mice. (A) Showing fold of increase in expression levels considering the expression in control as 1-fold; (B) IL-10, (C) TGF-β, (D) TNF-α, and (E) IFN-γ expression (relative to GAPDH) in aorta arches of mice treated with C5aR-P1 and C5aR-P2 either singly or in combination. (F) Showing fold of increasing or decreasing expression levels considering the expression in control as 1-fold. Values are means ± SEM from 5 mice per group. *P<0.05.

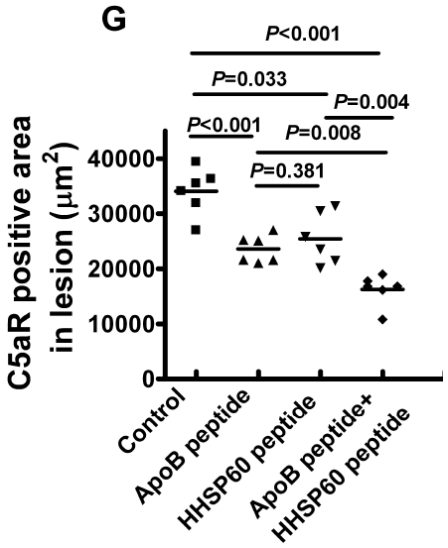
Figure IV. Analysis of peptide antigen epitope-induced specific $CD4^+$ T-cell proliferation

$CD4^+$ T cells isolated from the splenocytes of mice were incubated with the peptide at the dose of 0.01–10 μM/L in the presence of irradiated stimulator spleen cells. (A) $CD4^+$ T-cells from C5aR-P1-immunized mice stimulated with C5aR-P1 peptide and with C5aR-P2 peptide. (B) $CD4^+$ T-cells from C5aR-P2-immunized mice stimulated with C5aR-P2 peptide and with C5aR-P1 peptide. (C) $CD4^+$ T-cells from a combination of C5aR-P1 and C5aR-P2-immunized mice stimulated with either C5aR-P1 or C5aR-P2 peptides. (D) $CD4^+$ T-cells from KLH-treated mice stimulated with C5aR-P1 or C5aR-P2.

Figure I


A
C5aR
C5a
Combination
Magnified
KLH
C5aR-P1
C5aR-P2
C5aR-P1+P2
B
C5aR+cells per mm2 lesion
Control (KLH)
C5aR-P1
C5aR-P2
C5aR-P1+P2
C
C5a+ area in lesion (%)
D
Numbers of subset cells/mm2
C5aR+C5a+cells
C5aR+C5a-cells
E
Subset of C5aR+C5a+/- cells (%)
***


F
C5aR
C5a
C5a/C5aR
Control
ApoB-peptide
HHSP-peptide
ApoB+HHSP peptides


G
P<0.001
P=0.033
P=0.004
P<0.001
P=0.008
P=0.381
C5aR positive area in lesion (μm²)
40000
30000
20000
10000
0
Control
ApoB peptide
HHSP60 peptide
ApoB peptide+ HHSP60 peptide

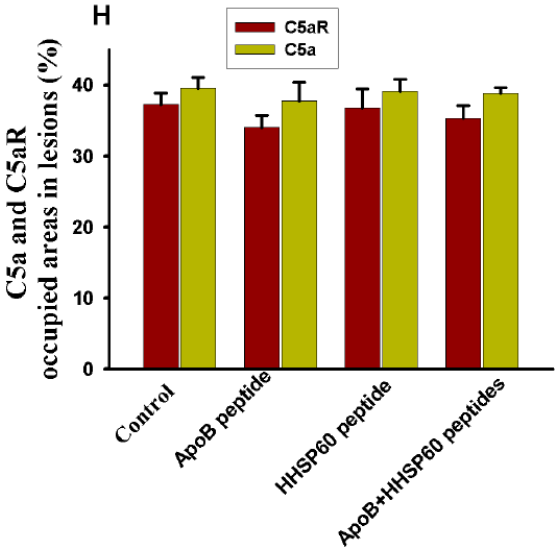

H
C5aR
C5a
C5a and C5aR occupied areas in lesions (%)
40
30
20
10
0
Control
ApoB peptide
HHSP60 peptide
ApoB+HHSP60 peptides

Figure II

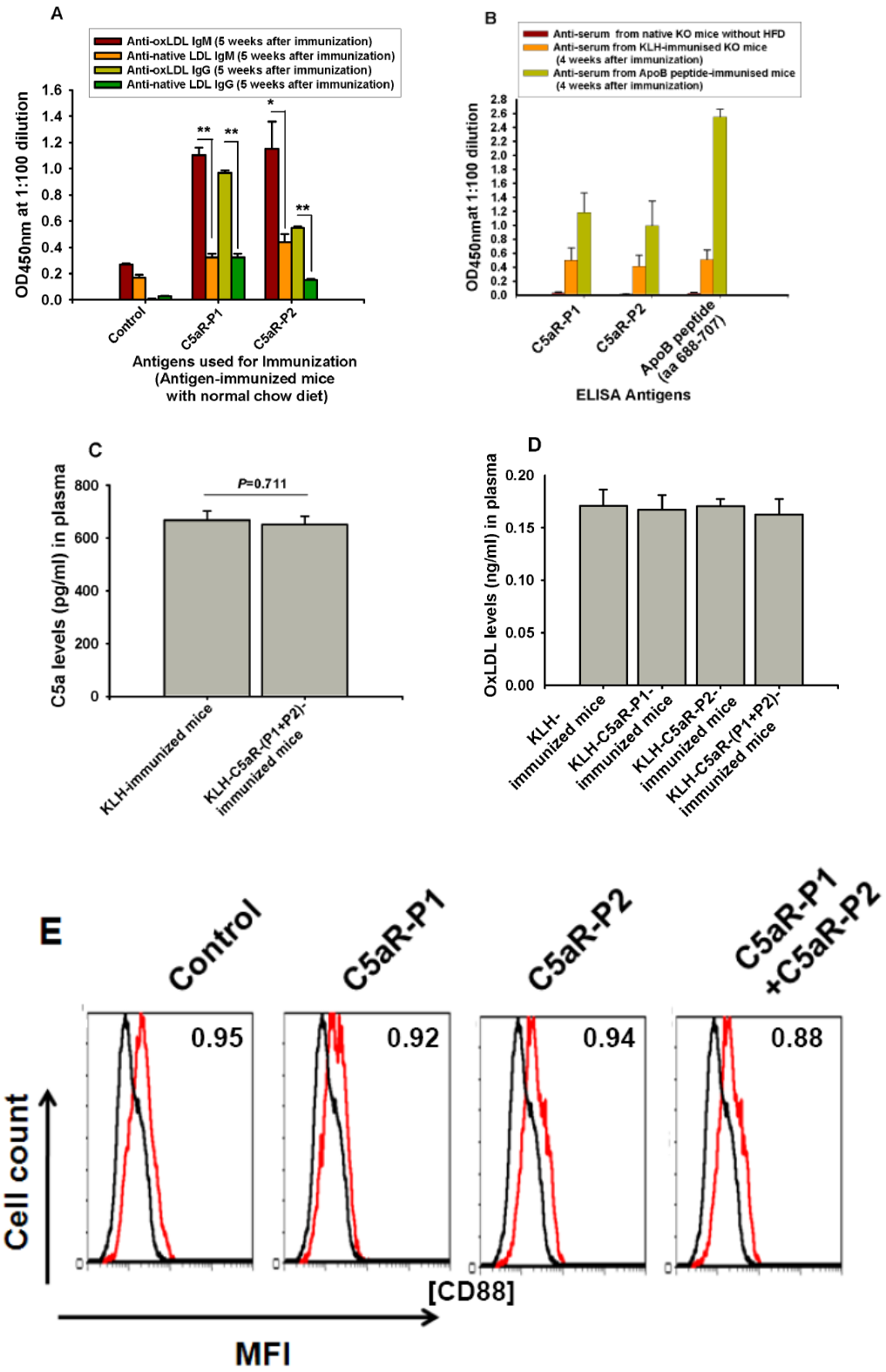

A
Anti-oxLDL IgM (5 weeks after immunization)
Anti-native LDL IgM (5 weeks after immunization)
Anti-oxLDL IgG (5 weeks after immunization)
Anti-native LDL IgG (5 weeks after immunization)
OD450nm at 1:100 dilution
Control
C5aR-P1
C5aR-P2
Antigens used for Immunization
(Antigen-immunized mice
with normal chow diet)
B
Anti-serum from native KO mice without HFD
Anti-serum from KLH-immunised KO mice
(4 weeks after immunization)
Anti-serum from ApoB peptide-immunised mice
(4 weeks after immunization)
OD450nmat 1:100 dilution
C5aR-P1
C5aR-P2
ApoB peptide
(aa 668-707)
ELISA Antigens
C
C5a levels (pg/ml) in plasma
P=0.711
KLH-immunized mice
KLH-C5aR-(P1+P2)-
immunized mice
D
OxLDL levels (ng/ml) in plasma
KLH-
immunized mice
KLH-C5aR-P1-
immunized mice
KLH-C5aR-P2-
immunized mice
KLH-C5aR-(P1+P2)-
immunized mice
E
Control
C5aR-P1
C5aR-P2
C5aR-P1
+C5aR-P2
0.95
0.92
0.94
0.88
Cell count
[CD88]
MFI

Figure III

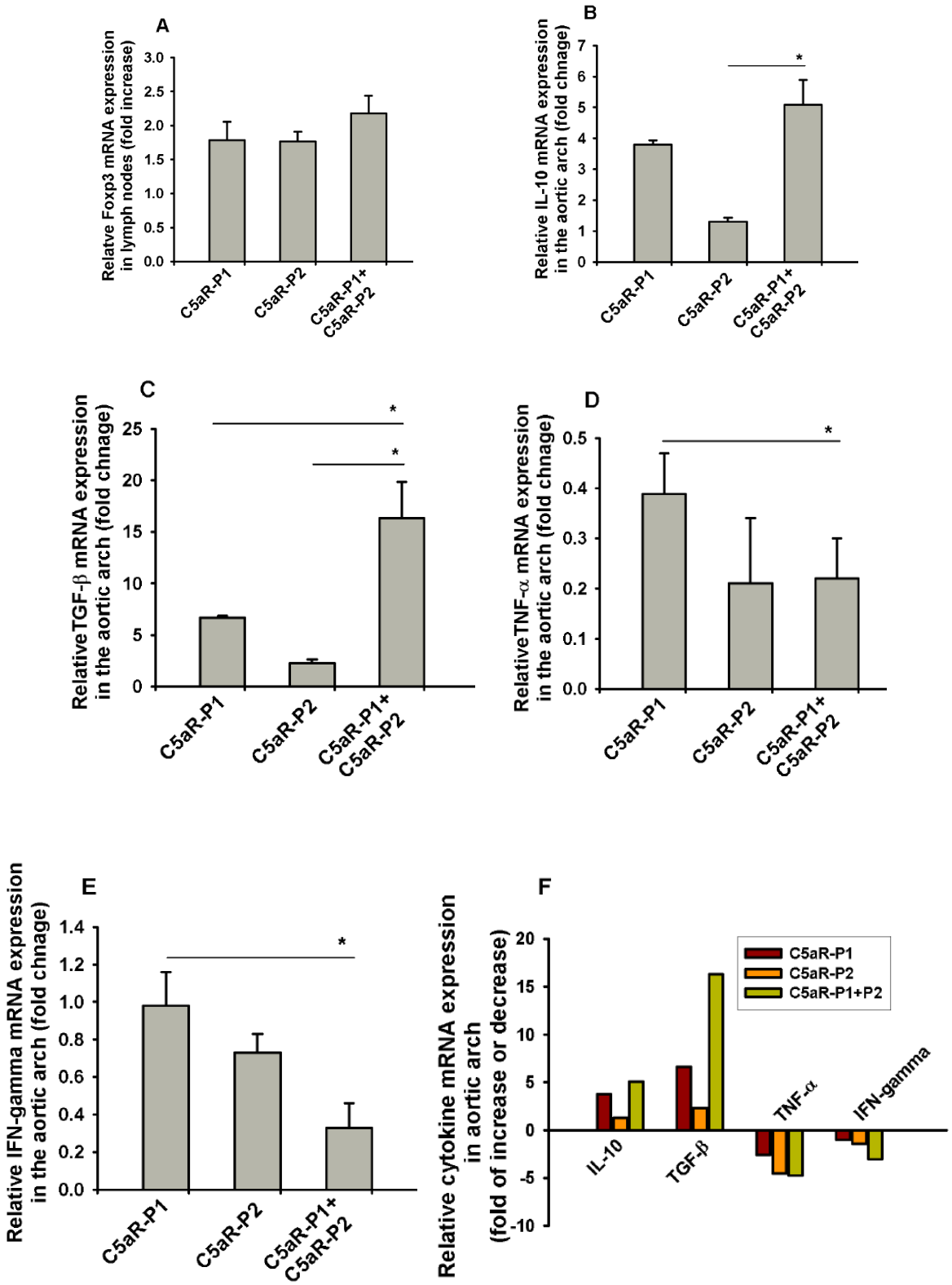

A
Relative Foxp3 mRNA expression in lymph nodes (fold increase)
3.0
2.5
2.0
1.5
1.0
0.5
0.0
C5aR-P1
C5aR-P2
C5aR-P1+ C5aR-P2
B
Relative IL-10 mRNA expression in the aortic arch (fold chnage)
7
6
5
4
3
2
1
0
*
C5aR-P1
C5aR-P2
C5aR-P1+ C5aR-P2
C
RelativeTGF-β mRNA expression in the aortic arch (fold chnage)
25
20
15
10
5
0
*
*
C5aR-P1
C5aR-P2
C5aR-P1+ C5aR-P2
D
RelativeTNF-α mRNA expression in the aortic arch (fold chnage)
0.5
0.4
0.3
0.2
0.1
0.0
*
C5aR-P1
C5aR-P2
C5aR-P1+ C5aR-P2
E
Relative IFN-gamma mRNA expression in the aortic arch (fold chnage)
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
*
C5aR-P1
C5aR-P2
C5aR-P1+ C5aR-P2
F
Relative cytokine mRNA expression in aortic arch (fold of increase or decrease)
20
15
10
5
0
-5
-10
C5aR-P1
C5aR-P2
C5aR-P1+P2
IL-10
TGF-β
TNF-α
IFN-gamma

Figure IV

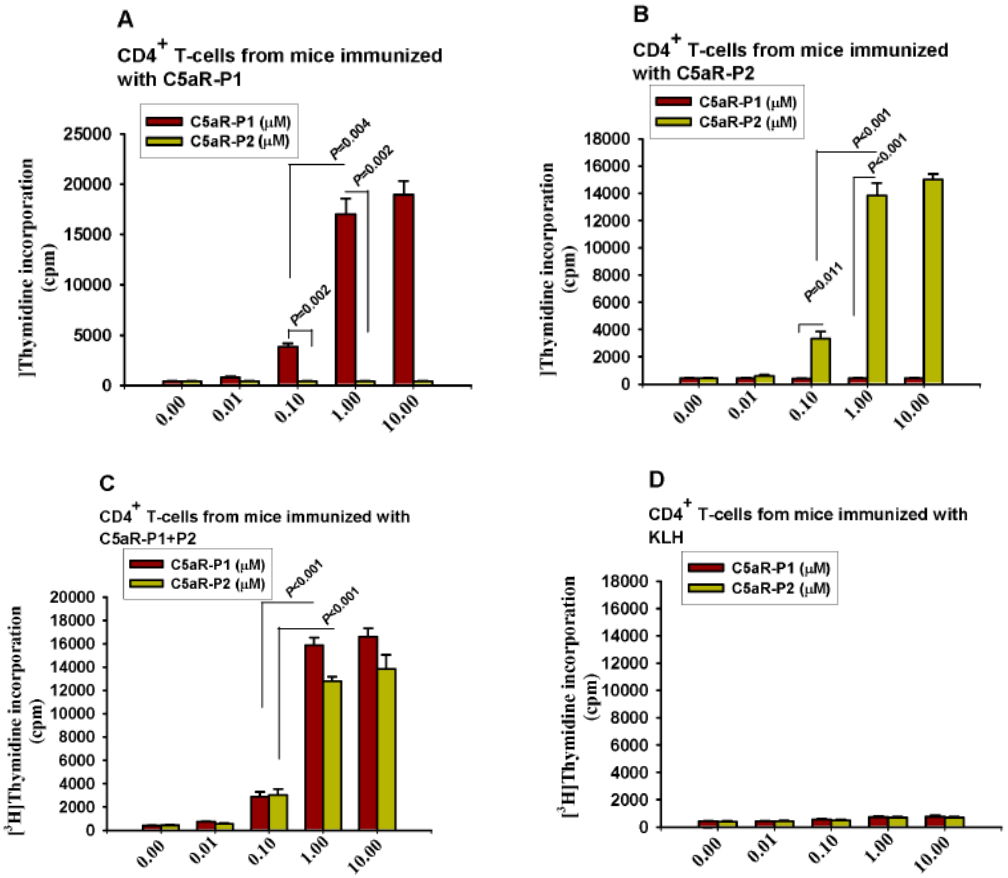

A
CD4+ T-cells from mice immunized with C5aR-P1
C5aR-P1 (μM)
C5aR-P2 (μM)
P=0.004
P=0.002
P=0.002
[Thymidine incorporation (cpm)
25000
20000
15000
10000
5000
0
0.00
0.01
0.10
1.00
10.00
B
CD4+ T-cells from mice immunized with C5aR-P2
C5aR-P1 (μM)
C5aR-P2 (μM)
P<0.001
P<0.001
P=0.011
[Thymidine incorporation (cpm)
18000
16000
14000
12000
10000
8000
6000
4000
2000
0
0.00
0.01
0.10
1.00
10.00
C
CD4+ T-cells from mice immunized with C5aR-P1+P2
C5aR-P1 (μM)
C5aR-P2 (μM)
P<0.001
P<0.001
[3H]Thymidine incorporation (cpm)
20000
18000
16000
14000
12000
10000
8000
6000
4000
2000
0
0.00
0.01
0.10
1.00
10.00
D
CD4+ T-cells fom mice immunized with KLH
C5aR-P1 (μM)
C5aR-P2 (μM)
[3H]Thymidine incorporation (cpm)
18000
16000
14000
12000
10000
8000
6000
4000
2000
0
0.00
0.01
0.10
1.00
10.00

Table 1. Lipid concentrations in plasma and body weight of peptide-immunized mice

Female mice					Calculated values	Body weight after feeding with HFD for 10 weeks
Immunization	**Diet (weeks)**	**Cholesterol (mmol/L)**	**TRI (mmol/L)**	**HDL (mmol/L)**	**LDL (mmol/L)**	**(g)**
KLH-C5aR-P1-immunized mice	**10**	**47.61**	**1.26**	**9.47**	**37.57**	**21.73±1.29**
KLH-C5aR-P2-immunized mice	**10**	**42.87**	**2.04**	**9.05**	**32.89**	**22.35±0.60**
KLH-C5aR-P1+KLH-C5aR-P2-immunized mice	**10**	**48.70**	**1.67**	**9.16**	**38.78**	**23.40±1.94**

Abbreviations are: HFD: high-fat diet; HDL: high-density lipoprotein; LDL: high-density lipoprotein; TRI: triglycerides.

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